COLLOID CHEMISTRY

THEORETICAL AND APPLIED

BY SELECTED INTERNATIONAL CONTRIBUTORS

COLLECTED AND EDITED BY
JEROME ALEXANDER

Preface

While technical men have not yet fully realized the importance of colloid and biological chemistry in industry, those biologists and physicians who are leading research in their sciences are keenly alive to the fact that without a knowledge of the colloidal behavior of living matter, they cannot do full justice to their own fields of endeavor. Depth of mental focus is what is now most needed by the physiologist, the biologist, and the physician, as a prerequisite to their best work; for they are dealing with exceedingly complex systems, where increasing complexity enormously magnifies the number of possible structures and relations. Below the intricacy of organization which the microscope reveals even in a single cell lie the bewildering phenomena of the colloidal zone, of molecular orientation and aggregation, of atomic groupings to form molecules, and of electronic aggregation to form atoms. These are not isolated happenings, but are interrelated parts of every phenomenon, and serve to make the whole. No single instrumental score constitutes a symphony, even though it may carry the main theme most of the time. The whole orchestra expresses the music of which the layered score is the written record.

Out of the inconceivably great number of possibilities in life processes, only relatively few regularly develop, because the results forthcoming are made vastly more probable by factors often going down to the very electronic configurations of atoms themselves. Every chemical, physical, biological, or physiological change follows as a consequence of the shifting about of material particles. In biological and medical phenomena, the units we strive to follow are very complex and are themselves continuously undergoing changes, so that it becomes difficult, if not impossible, to form a precise mechanical picture of everything that goes on. Nevertheless, we must approximate this picture if we would understand the view of Faraday that there is one fundamental kind of force and Tyndall's statement that in matter lies the potentiality and possibility of all life.

No simple explanation, formula, or group of formulas, will ever explain the behavior of even a single ameba. Few phenomena are as simple as are the scientific theories advanced to explain them. "Ordinary" observation "proves" that the earth is flat—it requires extraordinary observation, with reference to distant objects, to develop the truth. The danger of narrow mental range, and especially of adherence to some term or shibboleth of cloudy meaning, is brought out by Goethe, where the guileful Mephistopheles is trying to mislead the Student, saying:

> "Ich wünsche nicht, euch irre zu führen.
> Was diese Wissenschaft * betrifft,
> Es ist so schwer, den falschen Weg zu meiden,
> Es liegt in ihr so viel, verborg'nes Gift
> Und von der Arzenei ist's kaum zu unterscheiden.

* Goethe here refers to theology; but the principles involved apply just as well to any branch of science.

Am Besten ist's auch hier, wenn ihr nur Einen hört
Und auf des Meister's Worte schwort.
Im Ganzen—haltet euch an Worte!
Dann geht ihr durch die sich're Pforte
Zum Tempel der Gewissheit ein."

And when the Student objects to slavish following, and says:

"Doch ein Begriff muss bei dem Worte sein,"

Mephistopheles craftily replies:

"Schon gut! Nur muss man sich nicht allzu angstlich qualen;
Denn eben wo Begriffe fehlen,
Da stellt ein Wort zur rechten Zeit sich ein.
Mit Worten lasst sich trefflich streiten,
Mit Worten ein System bereiten,
An Worte lasst sich trefflich glauben,
Von einem Wort lasst sich kein Iota rauben."

In biology and medicine there are naturally many highly controversial questions involving colloid phenomena; and the Editor, entirely irrespective of his own leanings, has endeavored to secure several outstanding views on each of these questions, with the hope that there may develop ideas that will bring us nearer to the truth. For with science, as in every other field of endeavor, the point of importance for progress is not "*Who* is right?" but "*What* is right?"

When a great investigator establishes new facts and expounds new theories, there is a perfectly understandable human tendency for his assistants, students, and supporters to believe that every idea their master has advanced is correct and unalterable. They often fail properly to appreciate opposing evidence, and sometimes expend their efforts in *ad hoc* experiments primarily designed to support what they regard as accurate and orthodox views. They thus unwittingly become *scientific fundamentalists*. The danger in cases like this is that science may become *institutionalized*. On the other hand, we must recognize the immense value of liberal and intelligent cooperation and team work. Free-lance experimenters have often carried off the prize, and will continue to do so; but the University and research laboratories have a distinct advantage, providing they steer safely between the Scylla of fun-, damentalism and the Charybdis of scientific anarchism.

In looking over the papers in this volume, the reader will do well to differentiate between *experimental data* and the *theoretical conclusions or deductions* drawn from them. The former may be checked by repetition and examined critically for neglected factors—the latter may be opposed by alternative reasoning, by other existing data, or by new experiments. While a single inharmonious fact may necessitate the abandonment of a theory it opposes, modification and not total destruction of the theory may be the wisest course. The greater the height of knowledge to which we rise, the wider our horizon and the more comprehensive our theoretical view. The specialist has been jocularly described as one who knows more and more about less and less. But the true specialist should also be a *generalist*, and should couple a hawk-like perception of essential detail with the breadth of vision of the old-school "natural philosopher."

As in the preceding volume, no attempt is made here to pass on questions of priority, and the Editor does not vouch for the justice of any

claim or implication of priority. As the mass of publications increases, it becomes difficult if not impossible for each worker to know all that has been done; and frequently important work like that of Gibbs lies neglected and dormant until some one finds it and appreciates it. The fact that the same idea comes independently to different investigators, in different countries and at different times, is not to the discredit of any scientist.

A word may be said about the grouping of the papers. Following the introductory paper are two on artefacts; then several on physico-chemical matters, four on proteins, five on inorganic ferments and enzymes, six on protoplasm and cell structure, four on microörganisms, one on fertilization, two on plants, and the rest on medical topics with special papers on several diseases and on pharmacology. As an appendix is given the reprint of a paper with the views of which several contributors take issue. While it is obviously impossible to include everything in a single book, the field is sufficiently well covered to give the reader an idea of the ubiquity of colloid phenomena, which, while not the sole factors in life, nevertheless enter to a greater or less extent into every manifestation of life.

The Editor expresses his sincere thanks to those who have contributed papers, to the translators (whose names are given with the work translated), and to all others who have helped here and there with manuscript and proof. The papers show the close interrelation of various branches of biological science, and it is hoped that bringing these scattered notions together in one book will be a convenience to many and an aid to progress.

<div align="right">JEROME ALEXANDER</div>

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I

General Considerations

In most instances, it is not possible to give a readily intelligible explanation of a phenomenon exhibited by any large aggregation of matter, in terms of material units of a size much smaller than those directly involved in the phenomenon in question. It may be theoretically possible to explain all phenomena, even the behavior of an automobile, a plant, or an animal, by means of some super-formula involving the behavior and relations of two particulate units, the positive electron (or proton) and the negative electron. But when we consider the difficulties encountered in interpreting the behavior of even a single chemical atom in terms of protons and electrons, it may be readily admitted that such a super-formula, if ever established, would be beyond the grasp of the human mind. In investigating complicated material phenomena, the wisest method is to proceed step by step, first analyzing them into simpler components, and then explaining each lower and less complicated concept, as far as possible, in terms of what may already be quite complicated concepts of the *next* underlying stratum of material groups, until we reach our present null point in the electronic components of atoms.

Broadly speaking, the sum-total of the concepts involved in any material phenomenon, is a constant (P); and if we simplify or diminish the complexity of the *substance or matter units* (M) reckoned with, we must correspondingly complicate or increase the *behavior factor* (B). That is, since the phenomenon is a function of both M and B, we may write

$$P = f(M, B)$$

and any increase in the complexity of M involves a corresponding simplification in B, and *vice versa*.

The human mind cannot readily follow very complex behaviors and re-

lations of units. We are therefore prone to reduce the behavior factor to relatively simple terms, thus allowing the complexities necessary to complete the total concept of the phenomenon, to remain disregarded in the material units involved. However, it is essential that the material units be analyzed, and that we focus our mental microscopes, so to say, on successively lower and lower levels of material structure, through the whole depth of the masses concerned. The subjoined table, arranged in order of complexity and size, shows the main physical groupings or aggregations at present recognized as units in dealing with phenomena; and for the reader's convenience, there are set opposite, for comparison, the commonly used biological units, morphological and genetic, which are to be referred to in detail in Part II.

TABLE I

Physical Units	Approximate Diameters	Biological Units
Electron {positive	2×10^{-8} Å	
{negative	3.8×10^{-5} Å	
Atomic nucleus {H	2×10^{-5} Å	
{Au	4×10^{-7} Å	
Atoms	0.1–0.6 mμ	{Moleculobiont
Molecules	0.2–5 mμ	{ (Hypothetical simplest life)
Molecular groups	0.5–10 mμ	
Primary colloidal particle....	2–20 mμ	{ 20–70 mμ Gene (D. melanogaster)
Secondary colloidal particle ..	5–100 mμ	{ 50 ± mμ Bacteriophage
		{ 2 ± μ Coccus
Microscropically visible particle	over 100 mμ	{ 0.2–3.5 μ Chromosome (D. melanogaster) { 5–15 μ Nucleus (D. melanogaster) { 7–25 μ Cell (D. melanogaster)
Visible particle	over 50 μ	
Mass	over 1 mm.	2.5 mm Drosophila melanogaster
Planet (Earth)	13×10^4 km.	Equivalents
Solar system	5×10^9 km.	1 Å = 1 × 10⁻⁷ mm.
Star cluster	5×10^1 lt. yr.	1 mμ = 1 × 10⁻⁶ mm.
Galaxy	5×10^4 lt. yr.	1 μ = 1 × 10⁻³ mm.
Einsteinian Universe	2×10^9 lt. yr.	1 lt. yr. = 9.4627 × 10¹² km.

It must not be supposed that Nature draws sharp lines of demarcation between all these physical groupings of matter, so as to simplify the task of teacher or student. And furthermore, throughout the whole range of phenomena, we find evidence of the highly mobile and ubiquitous negative electron, sometimes neutralizing a plus charge or establishing a minus charge in a particulate mass, sometimes streaming in unbelievably enormous numbers to form what we term "an electric current" and its resulting magnetic field.

RESIDUAL FIELDS OF FORCE

To take what is perhaps the simplest case first, when a single proton, as nucleus, and a single negative electron have combined to form a hydrogen atom, we have evidence of marked residual attractions or residual free fields

of force, which endow the hydrogen atoms with a chemical attraction so great, that unless something else intervenes, they unite with each other to form hydrogen molecules (H_2), with liberation of much heat. Even then the hydrogen molecules possess a certain amount of residual attraction, which, however, does not become noticeable until the kinetic energy of the molecules is quieted by intense cooling.

In like manner, when protons and negative electrons unite to form atomic nuclei, and when the complex nuclei unite with negative electrons to form any of the chemical elements more complicated than hydrogen, we always find unsatisfied fields of force—generally very powerful ones, though in the so-called "inactive gases" (He, Ne, Ar, Kr, Xe, Rn) the fields are relatively weak.

As we follow up into material groupings of greater and still greater complexity, we observe the development of what to our intelligence seem to be new "forces" and new "laws," although these are, no doubt, dependent on and explainable in terms of more complex behavior or relations of the simpler underlying particulate units. The behavior of a crowd or of a nation cannot easily be predicated from the behavior of its individual members; as Rousseau remarked, "Man is good; but men are wicked." The expression "mob psychology" has a real meaning, even though it involves a confession of ignorance as to the integration of motives actuating the individuals of the mob.

GROUPS OF FORCES

In considering the nature of the forces operative on matter, we are struck by the apparent conflict of opinion between those who regard certain reactions as "chemical", and others who maintain them to be "physical". The remarks of P. V. Wells [*J. Wash. Acad. Sci.*, 9, 262 (1919)] are pertinent. Wells states:

"Science has arrived at a stage in its evolution where the classification between physics and chemistry appears artificial. Of course all classification is necessarily arbitrary and appears so especially at the boundaries between classes. There must, therefore, be confusion and difference of opinion among those who approach the study of what may perhaps be called *twin fields*, such as physical chemistry and chemical physics, from different points of view. The appropriate attitude in such matters seems to be to avoid the artificial issue by classification and nomenclature derived from a viewpoint common to both.

"In view of the historical significance, of the words 'physical' and 'chemical,' their use in classifying forces appears rather unnatural. A less artificial nomenclature is that derived from the fundamental theory of the constitution of matter common to both physics and chemistry, according to which forces are classified as (1) *molar*,[*] (2) *molecular*, (3) *atomic*, and (4) *electronic*. There is little anthropomorphic in these words, and they center the attention on the phenomena. The classification is qualitative in the sense that no quantitative relations of these forces to energy have yet been defined. The electron theory of atomic structure, however, dispels much of the vagueness surrounding many of the forces, and raises into further prominence the conviction of Faraday that there is but one fundamental type of force.

"Electronic forces may be defined as those which maintain the negative or valence electrons and the positive nucleus in equilibrium as a single system.[**] Similarly, atomic forces may be defined as those which maintain two or more atoms in equilibrium as a

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[*] *Molar* is derived from the Latin *moles*, a large mass. In the Century Dictionary *molar forces* are defined as those producing motions between large masses, *molecular forces* those between molecules, but which are insensible at sensible distance.

[**] The forces holding protons and electrons together in an atomic nucleus, are also electronic. *J. A. & C. B. B.*

single system; molecular forces as those which maintain two or more molecules in equilibrium as a single system; and molar forces those which maintain two or more masses in equilibrium as a single system. Each group of forces may be regarded as the residual fields of force remaining unsaturated in the smaller systems constituting the components of the system under consideration.

"Whatever the nature of the fields of force, the effect of neighboring systems would be expected to be more definite the more discrete the structure. Thus electronic forces are definitely characteristic of the nature of the element, showing the finite differences of the periodic system. Atomic forces show more continuity, only two distinct types occurring, corresponding to primary and secondary valence. These may be called *primary* and *secondary* atomic forces. Molecular systems have lost so much of their discreteness that combinations of molecules do not follow the laws of definite and multiple proportions. In such phenomena as molecular association and surface structure, the discreteness of atomic constitution begins to give place to statistical continuity. Moreover, even in these phenomena, the forces are relatively so weak that molecules are not regarded as permanently grouped together."

SURFACE SPECIFICITY

The statistical continuity of which Wells speaks, applies to an integration of the surface-mosaic of residual forces—to a relatively considerable surface area seen, so to say, from a distance. If, however, we closely consider a very limited surface area, outstanding patterns in the residual surface forces come into evidence; we find a high degree of *surface specificity*.

Even with crystals of comparatively simple chemical compounds, the various crystal faces show individual characteristics, due to the orientation within the crystal of the constituent atoms or molecules. The specific orientation creates outwardly directed fields of force which are definite in structural detail and intensity. The complexity of these fields grows with increasing complexity of the molecular and atomic units within the crystal. Similar considerations apply to the surfaces of non-crystalline groups, where the number of possible formations is much greater. It is interesting to note that the most marked effects of specificity, especially in a biological sense, are observable within the *range of colloidal dimensions*, where the surface areas involved lie between the tinier areas ruled by the more definite electronic or atomic forces, and the comparatively large areas dominated by statistical continuity.

The high surface-specificity of an area of colloidal dimensions enables the surface to *select* the material particles which will adhere to it, and to *direct* the position in which they tend to orient themselves at the interface. It sometimes even happens that the conditions under which a surface is formed, exerts a determining influence on the orientation of the molecules comprising the surface, and consequently on the nature of the residual fields of force outwardly directed; and this, in turn, governs the ability of the surface to hold and direct other molecules. Thus Devaux found that by floating lenses of molten fatty acids on hot water, and allowing them to cool slowly *in situ*, the surface of the solidified lens which had been cooled in contact with air, could *not* be wet by water; whereas the bottom surface which had cooled in contact with water, *could* be wet by water. It would appear that the hydrophile residual fields of force were turned outwards in the bottom surface of the solid lens, while the hydrophobe or "oily" hydrocarbon fields pointed outward in the differently oriented molecules of the top surface.

Since the activity of a catalyst is determined materially by its outwardly directed fields of force, a change in the catalyst surface, e.g., by chemical

attack from the outside or through re-orientation of the component atoms or atomic groups, will modify or even inhibit its activity. Polymerism, tautomerism, and lability are well known to chemists and physicists, as examples of re-arrangement within the molecular boundary. It is quite possible that the surface change of certain catalyst particles may be due to what may be called *electroversion,* whereby, following change in the nature of the milieu or change in temperature, certain atoms or atomic groups previously at the interior of the catalyst particle, move to the exterior and change places with other atoms or atomic groups or rotate so as to expose different outwardly directed surfaces and forces. Diagrammatically, a molecule modelled like Figure 1a, would, by electroversion, become like Figure 1b.

The experiment of Devaux, mentioned above, and the experiments of G. Friedel on the directive influence of crystal surfaces on mesomorphic substances (see section on Orientation by Crystals, page 112, in Vol. I of this
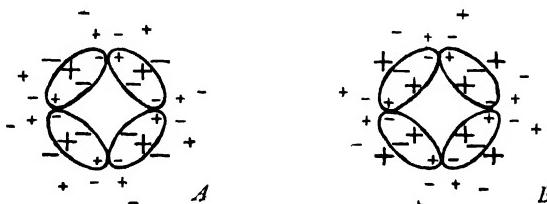
Fig. 1. -Diagrammatic representation of electroversion.

series), show how adjoining surfaces affect each other. The activity of enzymes is influenced by the reaction of the milieu—thus, pepsin works only in acid solutions, and ultramicroscopic observation of the addition of hydrochloric acid to dilute heated egg-albumen and pepsin (which had immediately aggregated into clumps of inactive ultramicrons), showed that the clumps immediately broke up, the ultramicrons began a violent Brownian motion, and the albumen particles quickly melted away, the field meanwhile growing brighter. (See J. Alexander, *J. Am. Chem. Soc.,* **32**, 680, 1910.)

There is much evidence (see e.g., the papers of R. Willstätter and of A. Fodor in this volume) to show that with enzymes, the maintenance of activity is dependent largely, if not entirely, on incidental "impurities", which may stabilize an effective orientation of a specific enzyme *substance,* or which may themselves furnish the active surface groups *to* this substance. In the latter case, the enzyme activity would depend on a specific and oriented adsorption, followed by such distortion of the fields of adsorbent and adsorbate, that a specific efficient catalyst area is presented to the milieu.

It seems evident that the orientation of molecules in the surface of a particle is a most important factor in determining what will be attracted to it and held by it—that is, in determining the behavior of the particle toward the milieu and toward other particles, including those of like kind. Thus, a slow reorientation of surface molecules may result in the aggregation and coagulation of sols and the breaking of emulsions. The mechanism whereby this occurs, following alteration of the orientation of surface molecules, need not be identical in all cases, but two consequences, either separately or in combination, seem to be important. First, adsorbed films of water or of other protective substances (protective colloids, ions, electrolytes) may be

sloughed off, thus permitting closer approach of the particles, which then enter the zone of extremely powerful mutual attractions; and second, the new orientations may of themselves be electrostatically more favorable to union of the particles. In many cases the naked or "stripped" particles will combine with each other, as do the fat globules in cream when churning breaks their protective protein films, or as molecules tend to adjust themselves to their common space lattice. In some cases, however, the combining tendency may be feeble, hampered, or effectually inhibited, and the velocity of combination will naturally be slow with particles whose size is large enough to reduce materially their Brownian or kinetic activity. We cannot gain a correct conception of the behavior of particles in these circumstances, by treating them as though they must be positive, or negative, or neutral. Any reasoning based on *statistical averages* is misleading here—we must consider the particles as *individuals*.

Since the activity of catalysts, including enzymes, seems determined by the orientation of their surface molecules, and since we shall later see that life involves numerous catalyses, it may very well be that continuance of life depends on certain orientations, and that death may follow a modification in surface orientation consequent on change in milieu, the action of X-rays, heat, ultraviolet light, etc., or simply the reorientations of ageing.

MECHANISM OF CATALYSIS

The concept of surface-specificity is intimately connected with most modern theories of catalysis, which agree in accepting the view that the reactions of catalysis take place within the interfacial zone.* On the other hand, catalysis is intimately associated with life processes; indeed, it seems to be the type of chemical action characteristic of life processes. It therefore becomes necessary to consider the mechanism by which catalysis proceeds. But this must be done with full realization that catalysis involves a large number of influencing factors, that we do not know all the factors, and that we cannot evaluate and predict the results of all those we do know. What might be laws in ideal systems, become *tendencies* in practical or actual systems. *Nature is a compromise of tendencies.*

CRITICAL VELOCITY

If a molecule approaches a surface slowly so that the two come within the range of molecular influence, there may be an attraction, a repulsion, or, more rarely, indifference. The tendency will be for the fields of force in both molecule and surface to produce changes in each other, but this is apt to be effective mainly in the approaching molecule, because in a solid surface the constituent molecules are rather rigidly held. Then again, since the fields about a molecule are generally not symmetrically distributed, much will depend on the way in which the molecule presents itself to the surface. Considering the effective areas of molecule and surface, the molecule will adhere to the surface only when the fields facing each other are such that the sum of the attractive forces predominates over the repulsions.

At the Toronto meeting of the British Association (1924), Sir Ernest Rutherford illustrated the influence of positively charged atomic nuclei on

* The importance of surface specificity is brought out in many of the papers in Vol. I of this series, especially those of Wm. D. Harkins, Irving Langmuir, Sir Wm. B. Hardy, and H. Freundlich.

positively charged alpha particles shot out by radium, by permitting a swing-
ing magnet (positive pole down) to pass near or over a fixed magnet (posi-
tive pole up).   In attempting to show that a direct hit would result in a re-
pulsion instead of a deflection, the swinging magnet was drawn too far back
on the first attempt, and acquired such a high velocity that it passed over
the fixed magnet, despite the magnetic opposition and correct aim.   The next
swing, at lower velocity, resulted in a repulsion.   It is quite evident that, in
an experiment of this kind, if the magnetic poles are of *opposite* polarity,
the velocity of the moving magnet will determine whether or not the two
will *cohere* or not.   The most favorable condition for cohesion is a direct
head-on meeting at a relatively low velocity, whereas an off-center approach
at relatively high velocity is unfavorable.   It seems obvious that, under a
given set of conditions, there is some velocity in excess of which there is
no fixation.*   Increase in temperature brings with it an increase in the
total number of encounters, per unit of time, between molecule and surface;
but it also changes the position of the maximum of the velocity distribution
curve, so that beyond a certain temperature limit, which should vary in each
case, the number of *successful* encounters per unit of time will decrease.   This
means that we may expect a certain temperature optimum for each catalytic
system, and as a matter of fact, this is very often observed.   Besides in-
creasing the activity of the molecules, as particulate units, heat affects their
internal structure, making electrons move more rapidly and perhaps jump to
outer quantum positions.   Practically, the particles move more rapidly, and
their fields may change in strength, arrangement, and location.   Similar con-
siderations apply to the catalyst surface, and to the molecules of the medium
in which the reacting molecules are distributed; as a rule heat reduces the
viscosity of a medium, which in turn enhances the kinetic activity of mole-
cules dispersed in it.

Catalyst surfaces also are influenced by temperature changes, and may
develop effective areas not previously present.   Too high a temperature may
melt a catalyst or cause surface flow, and thus ruin the catalyst.   If the cata-
lyst is colloidally dispersed, heat will tend to increase its particulate kinetic
activity distribution and the thermal optimum for each system of reactants
and catalysts should drop in the presence of a colloidal catalyst which has
active Brownian motion.   This is markedly the case with enzymes.

STEPS IN CONTINUOUS CATALYSIS

Let us now draw a picture of the successive steps occurring in a cata-
lytic *synthesis*, the catalyst being *A* and the result being the union of mole-
cules *B* and *C*.   A diagram representing this process is given in Figure 2.
Molecule *B* is fixed or adsorbed at the *effective* interfacial zone of the cata-
lyst *A*.   Its free fields of force adjacent to the surface will be largely bal-
anced by the opposing surface fields.   This neutralization leads to a redistri-
bution of the remaining electronic charges, with the result that the exterior
portion of molecule *B*, exposed to the dispersion medium, will have a different
electrostatic or electromagnetic configuration from what it had before fixa-
tion.   The molecule may then be able to make attachments which previously
it had not found possible.   Assuming that molecule *C* now approaches the
exposed surface of molecule *B* at not too great a velocity, and presents to

* See J. Alexander, *Science*, **65**, 62 (1927).

the exposed fields of *B* a field-mosaic that permits adhesion, molecules *C* and *B* will cohere. Immediately following this, there must be a complete re-shuffling or re-arrangement of *all* the fields of force involved, in the two molecules and in the catalytic surface as well. If the bond between the two molecules holds, while there is a break in the bond between the catalyst and
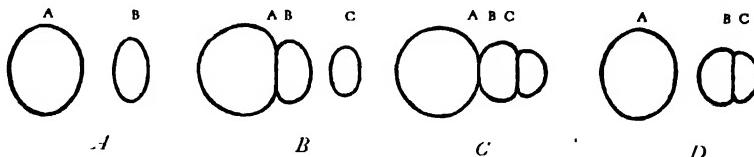
Fig. 2.—Diagrammatic representation of a simple type of continuous catalysis.

the now duplex molecule *BC*, the latter will be set free into the solution, its free fields undergoing readjustment in the operation. And the active catalytic area will be free to renew the process by adsorbing another molecule *B*. Quite similar would be the result if molecule *C* were fixed on the surface alongside of molecule *B*, but here more of the *surface* fields would be involved.

It is easy to see that the fixation of a *duplex* or *complex* molecule at an active surface may cause such a distortion or readjustment of fields, that first one part of the molecule would split off, and later the remaining portion would follow, as its fields changed. This would result in a catalytic *decomposition* or *analysis*.

"REPRODUCTIVE CATALYSIS"

In the catalytic processes we have been considering, the active surface is continually if intermittently cleared for renewed action, although with
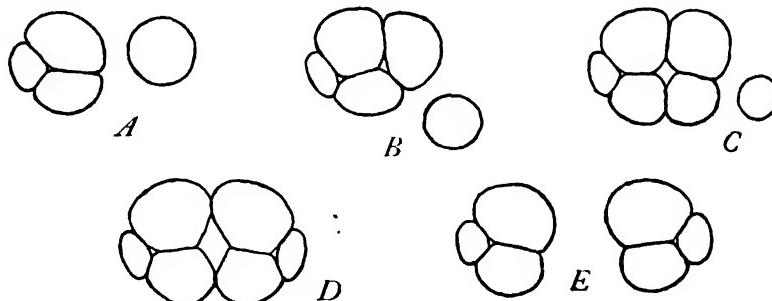
Fig. 3.—Diagrammatic representation of a simple type of reproductive catalysis (or autocatalysis)

time the active areas may become so distorted from continued use, or so occulted by the adsorption of impurities or "poisons", that they no longer function— that is, the catalyst becomes "worn out". But we have still to consider a whole series of possibilities in catalytic behavior, which lead to results quite different from those reached in the synthetic and analytic types of continuous catalysis outlined above. The molecules or molecular groups first fixed at the active catalytic areas, may cling there and continue to add

to their mass, new molecules joining on here and there, so that a relatively large and complicated group is formed before the active area is finally freed —if, indeed, it ever is freed at all. *If* the catalyst consists of a relatively small particulate unit, and *if* the groups assembled by it are condensed to form *an exact duplicate* of the catalyst unit, we would then have what may be justly called a *reproductive catalysis*—a true reproduction (Fig. 3).

A reproductive catalysis is, in fact, a kind of synthetic continuous catalysis, proceeding at comparatively a very slow rate, and generating more of the catalyzer.

The Primal Living Unit

Now suppose that we have a catalytic particle of *dual* activity, a particle which can, on part of its area, conduct a *continuous* catalysis (either analytic or synthetic), and can on another part of its area conduct a *reproductive* catalysis; and suppose that the substances formed by the continuous catalysis together with those existing in the milieu, are the very ones needed in the reproductive catalysis: *we would then*, if surrounding conditions were favorable, *have a self-perpetuating and self-duplicating catalytic particle,* which we must consider as *living*.

The question naturally arises: Is there such a relatively simple self-duplicating catalytic particle? Nothing of the kind has ever been identified or produced in the laboratory. Nevertheless we believe that such particles exist in countless numbers in living organisms, and probably constitute the basic living units in all biological structures. Our present knowledge indicates that in bacteriophages, in some ultrafilterable viruses, in genes or gene elements, we have biological units approaching this degree of simplicity.

How is the duplicate or "daughter" particle separated from the original or "mother" particle? This is what actually occurs, and there are several physico-chemical factors which seem to operate in this direction. Thus, following the adsorption or fixation of molecules or other particles at the "parent" interface, there would generally be a redistribution of their free surface fields, and following this reorientation the bond between "parent" and "daughter" particle might be weakened or even broken. Or something in the milieu might be then more powerfully adsorbed at the altered interface, and thus bring about what may be considered a deflocculation or peptization of the two particles.

In the case of chromosomes, the general rule seems to be that there is formed alongside of each gene its own exact duplicate; and the many hundreds of particulate units constituting the chromosome chain then form what acts like a *fiber*, the bonds being much stronger lengthwise of the chains than transversely, so that the then duplex chromosome splits lengthways. It is common knowledge that most crystals show preferential cleavage planes (mica is an outstanding instance); and X-ray examination of fibers shows that similar conditions obtain with them.*

that non-living things grow by *accretion*, whereas living things grow by *intussusception*. Thus, Huxley ("Anatomy of Invertebrates," p. 10, 1888) stated: "The increase of size which constitutes growth, is the result of a process of molecular intussusception, and therefore differs altogether from the process of growth by accretion, which may be observed in crystals." Accretion involves the addition of molecules or other particles to *exterior* surfaces only, whereas in intussusception the new matter is intermingled with the matter of the growing mass and tends to distend it or make it swell.

A little consideration, however, will show that what is intussusception at any given order of complexity of structure, is resolvable into accretional growth of component particles at some lower order of structural complexity. Thus, suppose we have a heap of tiny salt crystals in a supersaturated solution of the same salt, and that each little crystal begins to grow by the deposition of salt molecules on its exterior. Then, while each crystal would grow by accretion, the whole mass of crystals could be said to grow by intussusception. The reason why many felt that they saw a conflict in the two modes of growth, was because with growing crystals, attention was focused only on the large ones, while with growing living units not sufficiently small particles were considered.

With large crystals, the firmness of the space lattice prevents the penetration of adsorbed molecules into the interior; but with equally large living structures, as in fact with most colloids where the structural bonds are relatively weak, diffusion of solvent and other molecules or particles into the interior of the rather loosely meshed mass, takes place readily. The forces operative in growth, whether of crystals, colloids, or living matter, appear to be identical, namely the residual free fields of the particulate sub-units. And the unit which proves ultimately to be growing by accretion, may be of any order or size or complexity in the structural series, and may be crystalline, mesomorphic (see paper by G. Friedel in Vol. I of this series), or of random grouped structure.

Thus, intussusception in a body of a given order of complexity of structure, is resolvable into accretion at the surface of particles of some lower order, while conversely, accretion at the surface of particles forming a mass of higher order of complexity, determines the growth of this mass by intussusception.

As our knowledge of particulate matter develops, the mysteries of life and of vital phenomena become more comprehensible in terms of relatively simple concepts, providing we do not attempt to envisage the whole melée at once, but are content to consider successively portions of the field which present pictures whose comprehension is within the scope of our somewhat limited mental capabilities.

Particular attention must be drawn to three important papers by Dr. Leonard Thompson Troland, which have much in common with the views here advanced. Only by reproducing these papers in their entirety could full justice be done to them, but since space does not permit this, a few excerpts will be made which should serve to indicate the value of the papers and lead all interested to seek out and read the originals.

In the last of these papers [*The American Naturalist*, **41**, 326 (1917)], Troland says:

"As a matter of fact, in the school of the physical chemists there has been in preparation, since the days of Thomas Graham, a system of knowledge which, even in its

present unfinished form, has a most important and direct bearing upon mooted biological problems. This is the science of the *colloidal state*. The difficult abstractions and elaborate classificatory scheme, in terms of which the theory is now stated, will tend to be cleared up as our study of colloids comes definitely under the dominion of the general electro-molecular theory of matter. Intimate contact with the latter has already been established, indeed, through recent remarkable contributions by Langmuir, dealing with the atomic constitution of solids and liquids. It is to colloidal chemistry that we must look for answers to the large majority of the fundamental problems of vital activity. These answers will be slow in appearing, however, if we refuse to look.

"In fairness, it must of course be admitted that many biologists are keenly alive to the importance of the theory of matter, and especially of the theory of colloids, for the advancement of their science. However, possibly because the majority of these men are specialists in biochemistry, there seems to be a lack of coherent applications of modern physico-chemical ideas to the problems of evolution and heredity, which make up the heart of the biological mystery.

"It has for some years been my conviction that the conception of *enzyme action,* or of *specific catalysis,* provides a definite, general solution for all of the fundamental biological enigmas: the mysteries of the origin of living matter, of the source of variations, of the mechanism of heredity and ontogeny, and of general organic regulation. In this conception I believe we can find a single, synthetic answer to many, if not all, of the broad, outstanding problems of theoretical biology. It is an answer, moreover, which links these great biological phenomena directly with molecular physics, and perfects the unity not alone of biology, but of the whole system of physical science, by suggesting that what we call life is fundamentally a product of catalytic laws acting in colloidal systems of matter throughout the long periods of geologic time. This view implies no absurd attempt to reduce every element of vital activity to enzyme action, but it does involve a reference of all such activity to some enzyme action, however distantly removed from present activity in time or space, as a necessary first cause. Catalysis is essentially a determinative relationship, and *the enzyme theory of life,* as a general biological hypothesis, would claim that all intra-vital or 'hereditary' determination is, in the last analysis, catalytic."

The second excerpt is from Troland, 1914, p. 12:

"We have said that enzymes, and catalysts in general, have the power to assist in the production of specific chemical substances. Now there is no reason why the same enzyme should not aid in the formation of more than one substance and also why one of these substances should not be identical with the enzyme itself. A process of the last mentioned variety, in which the presence of a catalyzer in a chemical mixture favors the production of the catalyzer itself is known as *autocatalysis.* Many instances of auto-catalysis have been discovered in the field of inorganic chemistry so that it cannot be regarded as in any sense a vitalistic phenomenon, although we shall find the relationships which it involves to be indispensable in the chemical explanation of vital events. This will be made evident by the manner in which the conception of autocatalysis removes the difficulties faced by our theory of the origin of protoplasm. In order that the primitive life-substance, whose original formation we have described above, should be capable of continuous growth in all of its sub-divisions it is only necessary that the enzyme about which it centers should be autocatalytic, as well as effective in the production of primitive protoplasm. In this case each new globule which is formed from the original one may carry away with it a quantity of enzyme sufficient to permit its continuous growth and reproduction."

And on p. 18:   G ϶϶ 18

"Now the kind of molecule— or chemical substance—which is produced by any particular molecular collision must depend upon the nature of the molecules which originally collide and upon the force and direction of the collision, and these factors, in turn, so far as we are concerned, are referable to chance. Consequently we are forced to say that the production of the original life enzyme was a chance event. But once a single molecule of this enzyme was formed its very existence *increased the probability* that further molecules of the same sort would be produced in the same locality; this is the meaning of autocatalysis. The autocatalytic process thus established does not, however,

preclude the occurrence of further chance events of the same general kind as the one which produced the first enzyme; indeed, on account of the increase in chemical activity which it occasions the presence of the catalyzer decidedly favors such further events. Accordingly we have only to wait a sufficiently long time, and a second autocatalytic enzyme will appear, and this enzyme may furnish the basis of a new species of regulation in the substance which we have called primitive protoplasm, and which it was the sole duty of the original enzyme to synthetize from the oceanic solutes. The chemical reactions which are set up in the primitive living substance by the appearance of the second enzyme may be either favorable or prejudiced to its continued existence. In the former case the enzyme and the function which it subserves will be preserved by the action of natural selection; in the latter it will be destroyed together with all of those species of primitive protoplasm which contain it. In either event it constitutes a new biological variation, which, if favorable, provides the foundation of a new protoplasmic species. It would appear, then, that the *theory of enzymes provides a molecular basis for organic variation,* and as our discussion progresses it will become clear that this explanation is profoundly in harmony with the facts of biology, as well as with those of physics and chemistry."

The third excerpt is from Troland, 1917, p. 333:

"However, our explanation still remains somewhat special in its application, as in the majority of cases the products of catalysis do not adhere permanently to the catalytic surface. The extension of the explanation to cases of this sort is not difficult, since we have already seen that, even in the case of crystallization, the heat vibrations of the atoms are constantly throwing off molecular groups from the surface of the solid. As pointed out by Langmuir, the attraction between the surface and two molecular groups which have a strong affinity for each other may be less than the sum of the attractions of the surface for each of the groups, when separate. This is due to the 'closing up' or contraction of the fields of force of the groups as they come together. Hence combined groups of this sort will be more easily detached from the surface than will the uncombined groups, which will tend to be held in place until their mates fall into the right positions. The catalytic surface thus acts like an orienting sieve which on account of its special structure forces a chaotic crowd of individuals which come into contact with it, to fall into a special configuration. Many machines which accomplish exactly this effect are in use in the industries.

"Thus far we have dealt only with the mechanism of autocatalysis. Heterocatalysis is probably to be regarded as an extension of the process of autocatalysis. It is obvious that exact similarity of the force patterns of the catalyzing and catalyzed systems is not essential. Indeed, the catalytic effect which is based upon direct similarity of structure between the two systems should be much weaker than that which accompanies certain types of structural *correspondence,* such as that existing between a body and its mirror-image, or between a lock and a key. Special structural relations of this sort probably exist between stereochemical isomers, between acids and bases, etc. It is easily conceivable that the patterns of certain surfaces may be capable of distorting other special configurations which come under their influence, so that they fall into new equilibrium figures, without these figures being of necessity identical with those of the catalytic system. The general principles of the action, however, remain the same.

"Catalytic synthesis is a less common process in the laboratory than is destructive catalysis, but the laws of energy necessitate both effects, if either one is possible. Consequently the mechanism which we have described above must be an exactly reversible one, and must assist in the decomposition of molecular complexes as much as it aids in their synthesis. The deposition of the molecules to be decomposed upon the catalytic surface would naturally follow the same principles as those stated for simple polymerization. In this state of deposition the field forces of the crystal surface would inevitably have a tendency to open up the field of the deposited molecule, thus rendering it more unstable than before, in which condition the temperature vibrations of the system could break it up more easily than in the undeposited state."

Continuing on page 337, Troland says:

"It is evident, then, that the general theory of catalysis which has been outlined is applicable to *enzyme action,* which almost certainly depends upon the deposition or

adsorption of the reacting substances upon the surfaces of colloidal particles. Such adsorption, the molecular mechanism of which has been made very clear by Langmuir, will tend to be specific, and the more specific the more complex is the structure of the units making up the mosaic of the surface. Molecules, the field patterns of which fit closely into the fields of the surface, will tend to displace others having a cruder correspondence. This follows from either electrodynamics or thermodynamics, and obviously coincides with Fischer's classical conception of the lock and key relation between enzyme and substrate.

"It will be perceived that our theory of the catalytic process is simply a refinement and extension of the classical theory of 'intermediate compounds,' which has been proven true in so many instances. 'Adsorption compounds,' which play the principal role in enzyme action, do not differ dynamically from chemical compounds in general, since the forces causing adsorption are the same as those responsible for chemical union. Conversely, catalytic action in which the catalyst is in a molecular or unpolymerized state will not necessarily differ in its mechanism from that characteristic of enzymes or of metallic surfaces."

And on page 341:

"Although the fundamental life-property of the chromatin units is that of autocatalysis, it is necessary and legitimate to suppose that the majority of them sustain specific heterocatalytic relationships to reactions occurring in living matter. This is because nuclear material makes up a relatively small percentage of protoplasm, and because the reactions governed by enzymes are ordinarily heterocatalytic."

Finally, on page 346, Troland says:

"The enzyme theory of vital determination brings new life to the doctrine of evolution by accidental variation and natural selection, first, by showing that all fundamental variations should be discontinuous, or heterogenetic, as demanded by the mutation theory of De Vries, and second by revealing the exact mechanism of the production of these variations. The discontinuity follows from the existence of qualitative gaps between all specific chemical substances, such as those making up the system of genetic enzymes. The mechanism of production of any new chemical individual, i.e., the fortuitous encounter of the appropriate molecules with the right relative orientations and at the correct speeds (*vide supra*). The 'chance' nature of variation thus is made to depend upon that 'molecular chaos' which is so very familiar to all physicists, but the implications of which for biology have thus far been largely neglected."

In commenting on the high stability of the genetic material and its possible modes of transformation, it was stated [J. Alexander, *Science*, **56**, 325 (1922)]:

"The bio-colloids are so readily affected by salts, H-ion concentration (effective reaction), temperature, actinic (sun's rays) and traumatic (shaking, mechanical injury) effects, that it is more surprising that plants and animals should breed true, than that they should show variations. Therefore, although individuals may be much affected by such changes during their lives, it is evidently a rare occurrence that these changes are registered in the germ plasm by which alone they may be transmitted to offspring. The specificity of the germ plasm is evidently guarded by many factors, among which seem to be selective adsorption and differential diffusion of dissolved substances through its protecting walls or membranes. Nevertheless unusual influences must occasionally change it materially without destroying it, and along this line experiment may be directed."

Mutation and evolution are physical facts, however much we may differ about the causes underlying them. Beyond that given in Part II of this paper, space and time do not permit even a résumé of the many views advanced as to the nature and origin of life and the changes undergone by living forms. Several of the papers in this volume bear on these questions and

give references to the work of some of those who have treated these subjects. Practically everyone admits that life changes involve physical changes, and in considering the nature of these physical changes, we may safely relegate to the domain of metaphysics, where it belongs, the question as to whether or not there is within matter or without, a super-material force, power, or "entelechy" which dominates material behavior. What we *do* know is what our senses, experiments, reason, and experience tell us as to *how matter behaves.*

In an address delivered before the Australia Meeting of the British Association for the Advancement of Science, August, 1914, the late Professor William Bateson * in discussing "Heredity" said:

"Variation occurs as a definite event, often producing a sensibly discontinuous result; the succession of varieties comes to pass by the elevation and establishment of sporadic groups of individuals owing their origin to such isolated events; and the change which we see as nascent variation is often, perhaps always, one of loss. Modern research lends not the smallest encouragement or sanction to the view that gradual evolution occurs by the transformation of masses of individuals, though that fancy has fixed itself on popular imagination. The isolated events to which variation is due are evidently changes in the germinal tissues, probably in the manner in which they divide. It is likely that the occurrence of these variations is wholly irregular, and as to their causation we are absolutely without surmise or even plausible speculation. Distinct types once risen, no doubt a profusion of forms called species have been derived from them by simple crossing and subsequent recombination."

Since Bateson wrote this, we have had evidence that mutations and rearrangements of genes can be brought about by X-rays. (Muller, 1927b, 1928a, 1928b; Weinstein, 1928; Goodspeed, 1927. Goodspeed and Olsen, 1927.)

As our knowledge advances, it is even probable that we will learn of other agencies which may produce heritable changes in the usually well-protected egg and sperm. That heat is effective is already reported (Muller and Altenburg, 1923; Muller, 1927a). The query naturally suggests itself as to whether exposure to radioactive materials, which produce X-rays † and which are widely but irregularly distributed in nature, may not have some influence. Differential filtration of solar radiation and the so-called cosmic rays of Millikan might be mentioned as possibilities, although we have at present no knowledge along these lines.

Another aspect to be considered is the fact that over long periods of time, as the Boltzmann entropy-probability theorem indicates, there must be exceptionable kinetic excursions or stresses of molecules, which might leave permanent effects in labile systems. Thus, the view has been advanced that such a chance violent local disturbance set off the great explosion of ammonium nitrate in the Oppau disaster, and might also explain many otherwise incomprehensible magazine explosions. Where we are dealing with very large numbers of individuals, as is usually the case with living things, the chances of such an effective contingency are greatly increased.‡

The following excerpt is taken from a Master's Thesis entitled "The Importance and Trend of Recent Work on the Chemistry of Life and the Products of Life," by Jerome Alexander, presented early in 1899, but unpublished. Following a discussion of ferments and enzymes and the nature of their

* Reprinted as Smithsonian Publication 2396
† H. J. Bagg found heritable effects in mice after exposure to radium
‡ See, also, H. Freundlich, *Naturwiss*, 7, 832 (1919).

action, the question was taken up as to cells and microorganisms and the nature of their life:

"The enzymes and enzyme-like toxins we have just been considering, occur only in, or as the products of, living cells. Do they throw any light on the actual nature of life itself?

"After carefully considering the question, it appears to me that life is an enzyme-like continuous chemical process,* or rather a series of such processes. . . . That spontaneous generation actually did take place, no true evolutionist can deny. It is as necessary a tenet of his creed, as the fact that the life so formed underwent almost infinite change and development during aeons of time. Long as is the time during which the varied forms of life were evolved, how much longer must it have been from the Beginning, up to the time when, the necessary concordance of constitution and surroundings being established, there began the continuous chemical change we call life.

"With a much fuller meaning do we read the words of Dryden:

> "From Harmony, from heavenly Harmony
> This universal frame began:
> When Nature underneath a heap
> Of jarring atoms lay
> And could not heave her head,
> The tuneful voice was heard from high—
> Arise, ye more than dead!
> Then cold, and hot, and moist, and dry,
> In order to their stations leap,
> And Music's power obey.
> From Harmony, from heavenly Harmony
> This universal frame began:
> From harmony to harmony
> Through all the compass of its notes it ran,
> The diapason closing full in Man."

THE PARTICULATE UNITS OF LIFE

GENERAL RELATIONS AND PROPERTIES OF LIFE UNITS

The concepts relating to life have become highly varied and are rapidly changing in content as investigations proceed. Life needs more precise analysis as to its characteristics and more precise definition as to its line of demarcation from the non-living, in many directions. The method of analysis applied to the phenomena of life should be, as nearly as possible, that which has been so successful in the field of general physics, namely, the analysis of a given structure into units of simpler structure and the interpretation of the behavior of the whole in terms of the behavior of these components. This process results in a stratification of the analysis of the phenomenon, according to the units involved. The units themselves are seriated according to the order of complexity exhibited, each higher' order being essentially an aggregate of units of the order immediately below it in complexity. The series of units is thus a box-within-box series, as is well illustrated by the conceptual analysis of a given mass into molecules, of each molecule into atoms, and of each atom into positive and negative electrons. The units are of a particulate nature, and the forces which determine the patterns of their aggregations into units of the successively higher orders, are the residual forces unutilized in maintaining the internal patterns of the successively lower

* The text shows that a "continuous chemical process" is what we now call a catalyzed reaction.

orders. Thus, the molecules of a crystal are held in a space lattice by the forces that extend beyond the interior of each constituent molecule. Similarly, the forces holding the atoms in their places in a molecule are the un= utilized forces that are effective beyond the interior of the constituent atoms. The internal pattern of atoms is determined by the forces proceeding from the electronic components. Hence, we see that molar forces (the forces extending beyond the surface of a mass, also called the surface energies or fields of force of the mass), may be formulated in several equally valid ways: First, as the summation of the surface energies of the component molecules, minus (a) the molecular surface energies used in maintaining the internal pattern of the solid. Second, as the summation of the atomic surface energies, minus (b) that portion of the atomic surface energies used in maintaining the intramolecular structures and minus (a) the energies used in maintaining the intermolecular structure of the solid. Third, as the summation of the electronic surface energies, minus (c) the energies used in maintaining the intratomic bonds, minus (b) the intramolecular bonds, and minus (a) the intermolecular bonds. At present the structural analysis of matter has not penetrated with certitude below the electronic level. But it may be expected to do so, since the generalized procedure is the formulation of the properties of *any* given structural unit in terms of sub-units, and the forces proceeding from within these sub-units and relating them to one another as parts of the given unit. Already, observations on the quantum nature of radiation, and also speculations as to the mechanism of magnetism and gravity, furnish uncoordinated or poorly substantiated beginnings in the analysis of sub-electronic structure.

CLASSIFICATION OF MATTER BY TYPE. RANK OF STRUCTURAL UNITS

In the accepted classification of things, the primary division is into three *Kingdoms,* the Mineral, Vegetable and Animal. Of these, two Kingdoms are usually grouped as the "Kingdoms of living things" and the Mineral is recognized as the "Kingdom of the non-living". The distinction between the Animal and the Vegetable Kingdoms is ostensibly that of a wide divergence in properties; but the present diversity observed in properties is generally assumed to have resulted from variations within each of two evolutionary lines that have been separate since an early period in geological time. According to this same principle the Animal and Vegetable Kingdoms are each subdivided into *Phyla,* or groups within which an observed similarity of properties is assumed to be the result of similarity in descent. Moreover, it is generally assumed that the Kingdoms of life are themselves originations from the still more ancient Kingdom of the non-living. Thus, the generally accepted classification involves a high emphasis on the factor of *time.* The difference in properties are outcomes of divergences that have arisen along the evolutionary time-axis.

As a supplement to this classification, another may be proposed, which emphasizes to a greater degree another aspect of the same things, their *space-relations* or *structural units.* The primary division according to the type of unit, yields two Kingdoms, the *Abiontic* and the *Biontic.* Each of these Kingdoms would then be subdivided into *Ranks,* according to the order of complexity of the structural units revealed by analysis. In the opening paragraph of Part II, and in more detail in Part I, we have already referred to

the more significant *Ranks of abiontic units,* extending in a series below the mass to the present limit of assured penetration—the electronic Rank. In Part I we have also referred to a few of the Ranks above the mass, such as the Planet (Earth), the Solar-system, the Star-cluster, the Galaxy, and so on to the Einsteinian Universe, which is the limit of recognized aggregation.

In the following account we will use the term *biont* as a substitute for the usual but cumbersome phrase "individual living organism" (either plant or animal). We will follow the analysis of a typical biont, of the order of complexity of man, *into structural sub-units of successively lower rank,* and consider the relation of these structural units to the present and to the past series of bionts (free individuals).

THE CRITERION OF A LIVING UNIT

In the following account we shall support the thesis that one fundamental distinction between the living and the non-living type of unit is their characteristic method of increasing in numbers. *In non-living units it is by repeated production or repeated origination; in living units the first production only is an origination, thereafter increase in numbers is by self-reproduction.* The catalytic synthesis of molecules of H_2SO_4 may be used as an example of repeated production. Through the intermediation of the catalytic surface of platinum there occurs a condensation of raw materials (H_2O, SO_2, O_2, etc.), resulting in the production of H_2SO_4 and the liberation of by-products. This condensation is then repeated. The new molecules of H_2SO_4 have not arisen through the intermediation of previously existing H_2SO_4 molecules. The pattern of condensation of the new H_2SO_4 molecules has not been determined directly through the catalytic action of the old molecules, nor indirectly through the liberation of sub-units which progressively determined the development of H_2SO_4 molecules. The originations of molecules of the type H_2SO_4 are independent in point of time and usually also in point of space. Each is a discrete event, a new creation. *But in the increase in numbers of an existing living unit of a particular type, there is continuity both in space and in time.* Bionts of a given type are related in time as a continuous sequence—a chain of successive events. Living units of a given type are continuous in origination in space through the expansion of the space boundaries of the previously existing units, followed by subdivision of the contained space into sub-units, which in turn expand their peripheries. There may be secondary separation of the space sub-units, as in the reproduction of amoeba.

While it is maintained that no unit with specified characteristics can be considered living unless its increase in numbers is continuous in space and time, it is not maintained that all units which show such continuity are living. A snow-ball in rolling down a long slope (continuity in time) may increase its periphery and divide into sub-units (continuity in origination in space) without thereby being considered a living unit. Additional limitations as to the nature of the material occupying this space-time continuum must be recognized for living units, as distinct from non-living. The limits of these compositional differences can best be arrived at by starting with a unit, such as man, which by universal agreement is a living unit, and by continued analysis and extension in various directions, approach and pass over the boundary into the non-living.

THE UNIT-INDIVIDUAL

The unit referred to in the preceding paragraphs, is the whole which is characteristic of any order of complexity under consideration. For example, a man is a vital unit of a particular order of complexity or Rank. But upon analysis he is found to be an aggregate, and may be resolved into vital units of a lower order of complexity, namely, cells. The *unit-individuals* corresponding to the cellular Rank must, then, be considered in three aspects: (first), as to the manner in which the cell structure and activities control the structure and activities of the higher unit, and conversely the manner in which the cellular structure and activities are controlled by the sub-units of the cell. In accordance with the general physical procedure, the biological aim is to formulate the characteristics of any biological unit in terms of the next simpler units—of those units and of the residual forces that proceed beyond the interior of the sub-units, and relate them to one another as parts of the aggregate, either directly through interfacial actions, or indirectly through detached parts such as secretions or hormones. Equally important is to account (second) for the reproduction of a given vital unit in terms of the reproduction of its sub-units and (third) to account for the origination of the first example of any given modification of the unit, in terms of a modification in a sub-unit.

THE THREE BIOLOGIES

Each unit-individual thus has three Biologies: First, that which deals with the changes occurring within its individual space boundary and within its individual duration in time, its "life" as we say; second, that which deals with it as a member of a chain of such unit-individuals that are related through reproduction; and, third, that which deals with the origination of such a chain and also with its transformation into new chains through progressive and diversified branching of the given reproductive chain. These three biological fields may be briefly captioned *Metabolism, Reproduction* and *Evolution*.

METABOLIC BIOLOGY

Metabolic, or individual biology includes all the changes within the time-space extension of a given unit-individual, such as growth, differentiation and the constructive and destructive processes connected with its various activities. In more detail, metabolic life involves the intake of raw materials and energies or forces; the conversion, wherever necessary, of the raw materials into chemical or physical units combinable with those within the unit; the utilization, within the unit, of the materials and forces in the elaboration of structures; the functioning of the structures through rearrangement of the constituents, with release of new forces and formation of new products, which are further utilizable or are eliminated as waste. The basis of metabolic life is the continuance of the internal structure of the unit-individual. But this structure is labile and its persistence consists in an orderly series of developmental stages. So long as the structure of the unit remains intact and the environment favorable, life is manifested by the continuance of certain activities. If the environment becomes unfavorable, the structure may remain intact but the activities drop to a very low rate, a

condition present within seeds and in "latent life" generally. If the environment becomes still less favorable the activities may drop to the point of cessation and the structure may change so that the activities are not renewed even though the environment becomes favorable again. Metabolic success consists in the continuance of a unit individual, through a rate of constructive processes equal to or greater than the rate of destructive processes within the individual. The antonym of "alive" is "dead"—a term that implies that the unit-individual was once in metabolic activity based on a labile structural integrity, but is no longer so.

REPRODUCTIVE BIOLOGY

Reproductive biology, on the other hand, deals with change in the numbers of a unit-individual having a given set of characteristics. Increase in numbers is only through the intervention of the already existing units in the reproductive chain. Reproduction must also be explained in terms of the internal structure of the unit-individual of the preceding generation, that is, in terms of reproduction of sub-units. Reproductive success consists in the continuance of a reproductive chain through a rate of origination of characteristic individuals that is equal to or greater than the rate of dissolution of individuals of that same type.

EVOLUTIONARY BIOLOGY

Evolutionary biology is concerned with changes in the number of reproductive chains through the intervention of variation. Each new chain differs from its parental chain in at least one characteristic of its component individuals. The origination of a new chain is due to a change in some sub-unit of a component individual. Evolutionary success for a given chain consists in a rate of reproduction which maintains its parity with or gives it dominance over its parental chain.

In the account subsequent to this point we will deal very little with the metabolic aspect of life, but will proceed with the structural analysis into units and with the reproductive and evolutionary aspects.

PURE AND VARIANT REPRODUCTION OF THE UNIT-INDIVIDUAL

Reproduction, biologically speaking, is the process of producing new unit-individuals from those already existing. Typically, the new unit-individuals have characteristics identical with those of the parental form. One of the simplest types of reproduction, in living units of the Rank to which man belongs, is that exemplified by a new banana "tree", which arises from an underground shoot of the parental tree. Whenever a new unit-individual has a characteristic that is different from that of the parent, another process, *variation*, has taken place. Thus, a yellow-leaved banana tree may arise from a shoot of a green-leaved tree. If the yellow-leaved variant produces, through its shoots, green-leaved offspring, then the variant was due to a difference in the environment under which it developed as compared with the environment under which its parent and its offspring developed. Perhaps the spot at which the shoot emerged was too little exposed to the sun. If this be true, then shoots from the variant and from the parental type will

give identical results under identical exposures. But if the yellow-leaved variant produces yellow-leaved offspring under the same conditions as those under which the parental type is still producing green-leaved offspring, the variant was due to a change in the *internal* machinery of character production. That such changes in the internal machinery may be permanent, is shown by the numerous stable varieties of oranges that have been derived from variant branches of the parental Washington navel orange.

THE CELLULAR RANK OF UNIT-INDIVIDUALS

In the case of the banana tree, of a man, and of most of the easily visible plant and animal forms, the reproduced individual is composed of microscopic units, called *cells*. The reproduced individual is the end stage of a long and complex process of cellular growth, cellular division, and cellular differentiation. That is to say, reproduction of the individual is the resultant of the reproduction of constituent cells. Cellular reproduction may be "pure" reproduction, in which a given cell divides into two daughter cells and these eventually give rise to a reproductive chain or to a clone of progeny cells, all indistinguishable in characteristics from the parental cells. This pure type of cellular reproduction is exemplified also in artificial culture of certain tissue cells, and especially in cultures of the highly characteristic cell of sarcomas. Differences in the behavior of sister cells may arise from such facts as that cells whose surfaces are in mutual contact, or directed toward the interior of the group, are exposed to different conditions than the cells whose surfaces are largely in contact with the surrounding air, water or earth. Reproduction that gives variants due to environmental influence can be seen in the origination of colorless cells in a plant root. That this change is not permanent, is shown by the fact that such cells can give rise, under different conditions, to a whole new plant with all the different types of cells to be found therein. Finally, cell division may give rise to a variant in which the internal structure has been so altered that the parental type of cell no longer occurs in the progeny of the variant under normal conditions. The cancer cell arising from a normal tissue cell is such a variant, since its internal machinery for the production of cell characteristics has been altered permanently. This heritable alteration may be incited by any one of a number of agencies, namely, X-rays, chemical substances (e.g., in certain tars), bacteria or bacterial products, trauma and its chemical consequences, etc. (Winge, 1927).

From this point on in our analysis, *fluctuational variations due to environmental differences* will be disregarded.

GENETIC EFFECTS OF WHICH THE CELL IS THE UNIT

The effect of an initial difference in a cell upon the characteristics of a cell aggregate is clearly demonstrated in each case of hybridization between two pure-breeding species A and B. In the inbreeding of species A, eggcell A is joined to sperm-cell A, and the result is the development of an individual of adult type A. In the hybridization, egg-cell A is joined to a different sperm-cell, i.e., to sperm-cell B, and the result is the development of an adult different from A and from B. The origin of the mule from the union of horse and ass is a familiar example in point.

THE INTERNAL MACHINERY GOVERNING THE CHARACTERISTICS OF CELLS AND OF CELL AGGREGATES

What is this internal machinery governing the development of characteristics of cells and indirectly of cell aggregates? Microscopical examination of typical cells shows that each is composed of an outer layer, the *cytoplasm,* which constitutes the bulk of the cell, and of an inner vesicle, the *nucleus.* Cell division consists of the division of the nucleus into two sister nuclei and the cutting of the cytoplasm into two portions in the plane of separation of the nuclei. Each daughter nucleus is invested with its divided off portion of the cytoplasm.

A significant difference is observable between nuclear and cytoplasmic division. In each case division is preceded by growth in size and then by increase in numbers of certain components. But in the case of the nuclear components there is an exact doubling of each and an exact partition of the paired daughter components between the two daughter nuclei. The *components of the nucleus are the chromosomes,* generally cylindrical or spherical bodies. Each chromosome grows in diameter and then splits into two daughter chromosomes which are duplicates of each other. A special machinery, the diastral spindle, separates these daughter chromosomes from each other, and insures that each daughter nucleus gets one daughter chromosome from each original chromosome.

THE CYTOPLASMIC COMPONENTS

But in the case of the cytoplasmic elements there is generally less exactness in the increase in numbers and in the distribution on division. Many, perhaps the bulk of the cytoplasmic constituents, have no self-reproduction in the sense of one particulate structure dividing into two like parts which can grow and again reproduce. *The increase in number of most cytoplasmic components is through the repeated production of new particles by the same mechanism that produced the first few. The machinery governing their increase in numbers is not contained within themselves, but is elsewhere.* They are essentially manufactured products. As examples of this type of constituent may be mentioned pigment granules, excretory products, and a host of subvisible particles in the colloidal and molecular ranges. Among these bodies may be included very active substances, the *enzymes,* of which the cytoplasm carries a great variety. Cytoplasmic substances may interact with each other directly, or more typically through the intervention of enzymes; but their activities are best dealt with in their chemical, physico-chemical and colloidal aspects as part of metabolic life which is outside the scope of this paper.

The bulk of the cytoplasmic components do not possess characteristics that fit them to be sources of directing influences through successive generations of cells or cell aggregates. The first essential for a body as a possible integral part of the machinery governing the development of characteristics is that it shall itself be *autonymously self-reproducing.* The main cytoplasmic structures for which self-reproduction has been claimed are: chloroplasts and other plastids, centrioles, blepharoplasts, axial filaments of flagellae, Golgi bodies and chondriosomes.

Of these elements, chloroplasts are undoubtedly self-reproducing. How-

ever, they are probably not cell-organs; but are *Symbionts* (E. A. Minchin, 1912, p. 30), that is, elementary organisms of an algal nature that invaded the cytoplasm of the cell of the ancestors of the plants, and have since inhabited it. Probably a series of evolutionary changes have fitted these invaders much better for their intra-cellular existence. Moreover, chloroplasts are not essential components of cells, since they are absent from the cells of practically all animals. Similarly, centrioles and Golgi bodies are generally absent from plant cells, while blepharoplasts and axial filaments are especially characteristic of flagellate protozoa.

The chondriosomes may be universally present and may increase in number by fission. However, the fission is transverse and seems not to be meristic. The chondriosome must then be simple in structure, perhaps essentially an aggregate of a particular kind of molecule indefinitely repeated in number. A chondriosome might consist primarily of a single particle of a particular enzyme at whose surface is proceeding a rapid synthesis of the chondriosomic type of molecule. The chemical and physical properties of the synthesized molecules must then be such as to lead to the formation of an interfacial boundary between the continuous phase of the cytoplasm and the growing mass of synthesized molecules. The cylindrical form of the mass would indicate a strongly bipolar pattern for the fields of force of the synthesized molecules, and a consequent orientation of the molecules, as in the formation of asbestos crystals or fibrous bodies in general. A second possibility is that the source of the characteristic molecules of the chondriosome is not within the chondriosome, but quite outside. The enzymes responsible for the synthesis might be scattered throughout the continuous phase of the cytoplasm, or be localized at or near the place at which they originated—most probably within a chromosome. The chondriosome would then grow by a process analogous to crystallization * from a solution saturated for a certain molecule. The total of chondriosomic mass would continue to grow as long as the supply of specific molecules was maintained by their synthesis elsewhere. Autonymous self-reproduction would require that the increase in the number of enzyme molecules take place within the chondriosome and not elsewhere. The evidence with which we are acquainted does not seem conclusive that chondriosomes are autonomous self-reproducing bodies.

It has been suggested that some of the cytoplasmic constituents may be non-individualized as far as size, form and structure are concerned, but may be composed of self-perpetuating *material*. Instead of being self-perpetuating bodies, they are bodies of self-perpetuating material. Such a body would be an aggregate of specific molecules with non-specific quantity and arrangement. This conception would imply that in the growth of the body each new molecule of the specific substance was produced *within the body through the intervention of the previously existing specific molecules*. Thus,

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* It is not essential that this crystallization should mean exact placement in a three-dimensional space lattice As G. Friedel has shown (see his paper on Mesomorphic States of Matter in Vol. I of this series), molecules may undergo partial orientation in what have been termed "liquid crystals" Sometimes the molecules are held to one plane, but may move or sway in the plane, somewhat like skaters on ice, or sometimes molecules may be aggregated in chains along a line, but free to swing about this line, as keys strung on a rod or cord. When the vitreous humor of the eyes of mammals fluidifies, ultramicroscopic examination shows that the motion of the ultramicrons during the disintegration, is like that of pearls sliding along a thread on rod (Thiessen and Baurmann, "Nachr. d Gesellschaft zu Goettingen," *Math -phys Klasse*, June 30th, 1922. R. Zsigmondy, p. 819 of Vol. I of this series.)

Aceto-carmine preparations of the ovaries of Drosophila, yield a rich growth of filamentous or moniliform liquid crystals of microscopic size when the concentration of the extracted substances (probably lipins) is increased by slow evaporation of the acetic acid.

such a body would grow by the autocatalytic synthesis of molecules, as developed in detail in Part I of this paper.

The second essential for a body as a possible integral part of the machinery governing the development of the characteristics of the aggregate of which it is a component, is *inclusion* in each such aggregate. We have seen that generally the cytoplasm of a cell is simply cut through into two portions with little regard for quantitative or qualitative exactness in the partitioning of any given type of component. If a self-reproducing cytoplasmic component fails to be included in a cut-off portion as at least one unit-individual, then all cells and cell-aggregates descended from that portion, if viable, will lack the controlling effect of that body. Such an eventuality is a real possibility, but is the more remote the more numerous are the unit-individuals normally present, and the more uniformly they are distributed through the cytoplasmic mass.

The complex bodies of the higher plants and animals have come from a single cell, the fertilized egg, by an orderly series of developmental stages. The unfertilized egg—the bridge between the maternal soma and the soma of the offspring—is especially rich in cytoplasm. But the sperm cell, when it is examined microscopically, is seen to consist of little more than a nucleus. In the process of forming sperms in the higher plants, and, especially in the higher animals, nearly all of the cytoplasm is sloughed off and discarded. The bridge from the paternal soma to the soma of the offspring is thus a very slender one as far as the passage of self-reproducing cytoplasmic components is concerned.

The vast researches of modern experimental embryology and heredity have shown that, in general, the effect of the sperm is as great as that of the egg upon the characteristics of the *adult* offspring. In the early stages of embryonic development the effect of the maternal parent is generally greater than that of the paternal, but this excess effect is greatest at first and becomes progressively less as development proceeds. There is a genuine residue of maternal effect in the adult stage in several specific instances of differential inheritance. The most definite of these are in the higher plants and are demonstrably due to plastid transmission. But it is not impossible that cytoplasmic units other than the plastids may be the determiners of hereditary differences in a fringe of rather obscure cases. However, the total share of the cytoplasmic elements in governing differential heredity has been proved by genetical studies to be proportionately a very slight one. The controlling mechanism of the cell resides mainly in the nucleus, while the cytoplasm is predominantly controlled in its activities.*

EFFECTS IN HEREDITY DUE TO CHANGES OF WHICH THE NUCLEUS IS THE UNIT

Several phenomena in heredity afford direct demonstration of effects upon the characteristics of cells and of cell-aggregates, due to changes of which the nucleus is the unit. In these cases the change is generally not in number of nuclei, for two nuclei within a cell ordinarily fuse to form one. The total nuclear contents of cells with two nuclei fused would nevertheless have been doubled, and correspondingly the nuclear volume would be approxi-

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* The cytoplasmic components are treated in greater extension by Prof. E. B. Wilson in a paper in this volume.

mately doubled. The direct effect upon the cell is that the cytoplasm grows correspondingly to a double volume, or to a volume somewhat smaller than this, rather in the ratio of increase in nuclear surface area than in nuclear volume. This greater cell growth has a direct effect upon the cell aggregate. If the doubling of nuclear content occurs in the root tissues of the tomato or of the plant *Crepis*, the result is a lump of coarse but normally functioning tissue. If the doubling occurs in the root of the sugar beet, the lump grows irregularly and rapidly to form the characteristic "crown-gall", analogous to cancer tissue (Winge, 1927). Finally, if the doubling occurs early enough so that all the embryonic cells are affected, then the individual is also larger in size and coarser in structure. The converse change —halving of the nuclear contents—also occurs, with inverse effects upon the characteristics of cells and of cell aggregates.

The Nucleus as an Aggregate of Chromosomes

The work of the cytologists * has shown that *the nucleus is, in a particularly clear way, an aggregate, resulting from the coalescence of sub-units, the chromosomes.* In a cell that is about to divide (see Fig. 4F, for general relations only), the nucleus is seen to consist of a round vesicle filled with "fluid", in which are suspended cylindrical or rounded bodies—the chromosomes. These chromosomes soon become arranged in a single plane—the "metaphase plate". Meanwhile, the nuclear wall—the boundary of the vesicle —has disintegrated and the fluid of the nucleus becomes continuous with the general cytoplasm. Each chromosome divides longitudinally into two daughter chromosomes, which separate. Two daughter groups are formed, each containing a daughter duplicate of each of the original chromosomes. *At this stage no nucleus exists, only discrete chromosomes which are present in two groups, the "anaphase" groups, in the cytoplasm.* As the anaphase chromosomes move apart, the cytoplasm draws out into a more cylindrical form and eventually divides into two parts, each part containing one of the two groups of chromosomes. The chromosomes of each group draw closer together ("telophase" stage), with the gradual elimination of most of the cytoplasmic substance from between them. Each chromosome has a definite boundary or interface between its substance and the substance of the cytoplasm. Now occurs a growth of the individual chromosomes, with further elimination from between them of the substance of the cytoplasmic matrix. The surface of two telophase chromosomes come into contact and presently the interfacial boundary dissolves, as when two oil droplets of an emulsion touch and fuse. But in the case of the chromosomes there is no thoroughgoing mixture of the chromosome contents. It is as though each chromosome has a permanent *core*, and as though the growth of the telophase chromosomes preliminary to this fusion, consists predominantly in an increase in the amount of a semi-fluid colloid surrounding this core. As will appear later in this account, the core of the telophase chromosome seems to be a long slender thread disposed in rough cork-screw fashion within this colloid, but generally invisible at this stage. The spiral is loose and open, with the turns nearly reaching the periphery of the chromosome colloid.

The *pellicle* of the chromosome may be interpreted as the surface film

* The standard reference work on cell organization, very wide in scope and thoroughly illustrated, is that of Prof. E. B. Wilson (see Bibliography).

of this colloid, probably made more definite by adsorption or by precipitation at the interface between the intrachromosomal colloid and the general cytoplasmic matrix (Seifriz, 1921). As soon as the surfaces of the two telophase chromosomes touch, a two-layered interfacial film is between the colloidal contents of the two chromosomes. Presently the intervening film is broken down, and the two bodies of colloid flow together with a continuous film enclosing the two cores, much as in the case of fused oil droplets in an emulsion. The breaking down of the intervening film is probably to be explained on the ground of a very high similarity in the chemical composition of the colloid mixtures within the different chromosomes. A substance called *chromatin* seems to be the most characteristic and universally abundant ingredient of chromosomal colloids. When the pellicle of the chromosome no longer has the chromatin colloid on one side and the general cytoplasm on the other, the conditions that led to the origination of, and that maintained the existence of the film, are removed throughout the area of contact of the two chromosomes. Upon dissolution of the film, the colloid contents of the two chromosomes are brought into direct contact, and fuse, since they have only slight surface tension against one another. The coalescence is not simply between two contiguous chromosomes: it proceeds until all the chromosome cores are within a common film. This pellicle constitutes the new nuclear membrane of the "interphase" nucleus (Fig. 4A), and is derived from the surface films of the individual chromosomes. Thus, a genetically continuous pellicle is always interposed between the general cytoplasm and the chromosomal contents. The semi-fluid colloids which accumulate between the core and the surface film of each chromosome, are now mingled to form the nuclear sap of the interphase nucleus. The chromosome cores maintain their integrity, and even, to a surprisingly great extent, their original positions and orientations within the interphase nucleus.

Study of the chromosomes at the stage when they are lined up in the flat "metaphase" plate, preparatory to division, has shown that they are present in definite number. This number is characteristic of the nuclei of the individual or even of all the individuals of that species or genus; for example, 14 chromosomes in the nuclei of garden peas and 48 chromosomes in the nuclei of man. Furthermore the different chromosomes of a given species may possess individual peculiarities which distinguish one chromosome from another. Among these distinguishing features are characteristic diameter, length (varying from a sphere to a cylinder with a length perhaps thirty times its diameter), segmentation, shape (whether straight, J-shaped or V-shaped), attachment of spindle fibers, and position in the metaphase plate. The chromosomes usually exist in pairs, one of each definite kind having come from each parent. The egg contributes one "haplid" (that is, a group containing one of each kind of chromosome), and the sperm another haplid. Thus, the nucleus of the fertilized egg and the nuclei of cells in a multicellular adult, are dual in structure.

EFFECTS ON HEREDITY DUE TO CHANGES OF WHICH THE CHROMOSOME IS THE UNIT

Very abundant and clear is the cytological and genetical evidence which shows that a change within the nucleus, by the increase or decrease in the number of a particular chromosome, leads to hereditary effects upon all cells

and cell-aggregates that carry the changed nucleus. Thus the absence of one chromosome of the pair of "X"-chromosomes of the nuclei of the fertilized egg of the grasshopper leads to the development of the male individual. The presence of an extra chromosome of a particular kind (making three of that kind, while the other kinds are represented by two only) in the fertilized egg of the evening primrose, leads to the development of a particular variety called "lata" (Lutz, 1912; Gates, 1912). In the jimson weed, *Datura*, a dozen such special varieties have been demonstrated, each of which contains an extra chromosome of a particular one of the 12 kinds normally present (Blakeslee, 1922). In the fruit-fly, *Drosophila*, there are known (Bridges, 1925) analogous special varieties, due to changes in the relative numbers of the chromosomes of the zygote.* The specially favorable nature of this insect for studies in heredity, has enabled such "chromosomal mutants" to be analyzed further and to be expressed in terms of units of a still lower order of complexity—namely, the *genes*, as will be shown presently.

The Gene as a Unit in Heredity

The recognition of the gene as a unit came primarily through studies in heredity, initiated by Mendel (Mendel, 1865) some eighty years ago. Mendel found that in the garden pea, a cross between a white-flowered variety and the usual purple-flowered variety, gave only purple-flowered offspring. But if these offspring were inbred, the next generation (F_2) consisted of purples and whites in the ratio of 3 to 1. The explanation advanced was that *the white characteristic depended upon a germinal sub-unit* (now called a "gene") *which remained intact in the hybrid and which emerged in pure form in half of the gametes.* The remaining half of the gametes received a germinal representative of purple-flower, which representative had likewise remained intact and pure. Random unions between eggs that are purple-bearing or white-bearing with pollen that are either purple-bearing or white-bearing, would result in one purple-purple, like the original purple: 2 purples-whites, like the F_1 hybrids; 1 white-white, like the original white. Mendel found that all the varietal differences with which he worked in peas, behaved in the same manner, giving sharp re-separation in 3:1 ratio, for the types originally crossed. Furthermore, when he crossed plants which had differed in two separate varietal characteristics, as white or purple flowers and short or long stems, the re-emergence was in a 9:3:3:1 ratio. This meant that in the formation of the germ cells of the doubly hybrid plant, *the units of the two contrasting pairs of characters had been able to fall out of their original combination, and to recombine at random.*

It was pointed out later that the behavior of the paired germinal units could be explained in terms of known cytological units, the *paired chromosomes* (Sutton, 1902). Thus, the germinal unit for white-flower could be in one chromosome of a particular pair, and for purple-flower in the other. Similarly, the random assortment that held for crosses involving two or more pairs of characters, might mean that *a separate chromosome pair was involved for each character pair which showed assortment independent of the other character pairs.* The random assortment of the chromosomes presumably occurred during the formation of the gametes.

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* "Zygote" is the technical name for the fertilized egg cell formed by the union of the two gametes, the egg and the sperm. By extension, it is sometimes used for the embryo or adult that develops from the zygote.

. In Drosophila the number of character pairs soon became much greater than the number of chromosome pairs, which was four. This necessitates that each chromosome should carry the germinal units of several character pairs. In other words, the germinal unit could not be a whole chromosome but must be some *sub-unit of the chromosome*.

THE CHROMOSOME AS AN AGGREGATE OF GENES

The phenomenon of *"linkage,"* i.e., non-random distribution in a cross involving two or more character pairs, was discovered (Bateson and Punnett, 1905). It was suggested that linkage indicates that the character pairs involved in the linkage depend upon germinal units carried by *the same chromosome pair* (Lock, 1906). A high departure from randomness, i.e., *strong linkage,* was interpreted as due to *positions for the two germinal units close together along the common chromosome* (Morgan, 1911). The recombination of genes is carried out in blocks, supposedly by the breaking across of the two homologous chromosomes and the reunion of the fragments in new combinations. This process of breaking and trans-union is called *crossing-over* (Sturtevant, 1913). The farther apart along the chromosomes the loci of two genes are, the greater should be the number of crossings-over that would occur between them per hundred gametes. Taking the number of crossings-over as varying directly with the distance (Sturtevant, 1913) *maps* were made which showed for each of the linkage groups of Drosophila, *the arrangement of the genes within the group.* It was found that the genes of a given group are arranged in a *linear sequence like a string.* Each gene occupies a *definite position* in the gene sequence, presumably corresponding to a definite position at some point along the chromosome. Finally, proof was forthcoming that the *Mendelian genes and the gene-strings are in fact contained within the chromosomes* (Bridges, 1916, 1921, 1925), *and that the maps represent the placement of genes along the gene-string and along the chromosome* (Bridges, 1917). This proof came from aberrant distributions of Mendelian genes, which distributions were shown to be due to corresponding aberrant distributions of chromosomes.

THE GENE-STRING AND THE CHROMONEMA

While the genetical proof is now complete that the gene-string is carried within the chromosome and that the Mendelian genes are in reality sub-units of the chromosome, cytological investigation of the chromosome has not yet revealed structural sub-units that can be directly homologized with the gene-string and the gene. This is not to be wondered at, since calculations for the number of genes in Drosophila give a minimum number of about 2000 (Morgan, 1922), and suggest that 20,000 is a more probable number. Calculations for the average size of the gene (Morgan, 1922) gives values of from 70 to 20 mμ. An object of this size is probably not visible in the ordinary microscope, and it is therefore probable that no structure so far seen is actually a gene. Similarly, no structure thus far seen is probably itself a gene-string. From the cytological side, as opposed to the genetical, the whole question of detail of chromosome structure is inextricably entangled with the interpretation of "chromatin," which will be considered later in some detail. Moreover, in living material the finer details do not have enough optical distinctness to
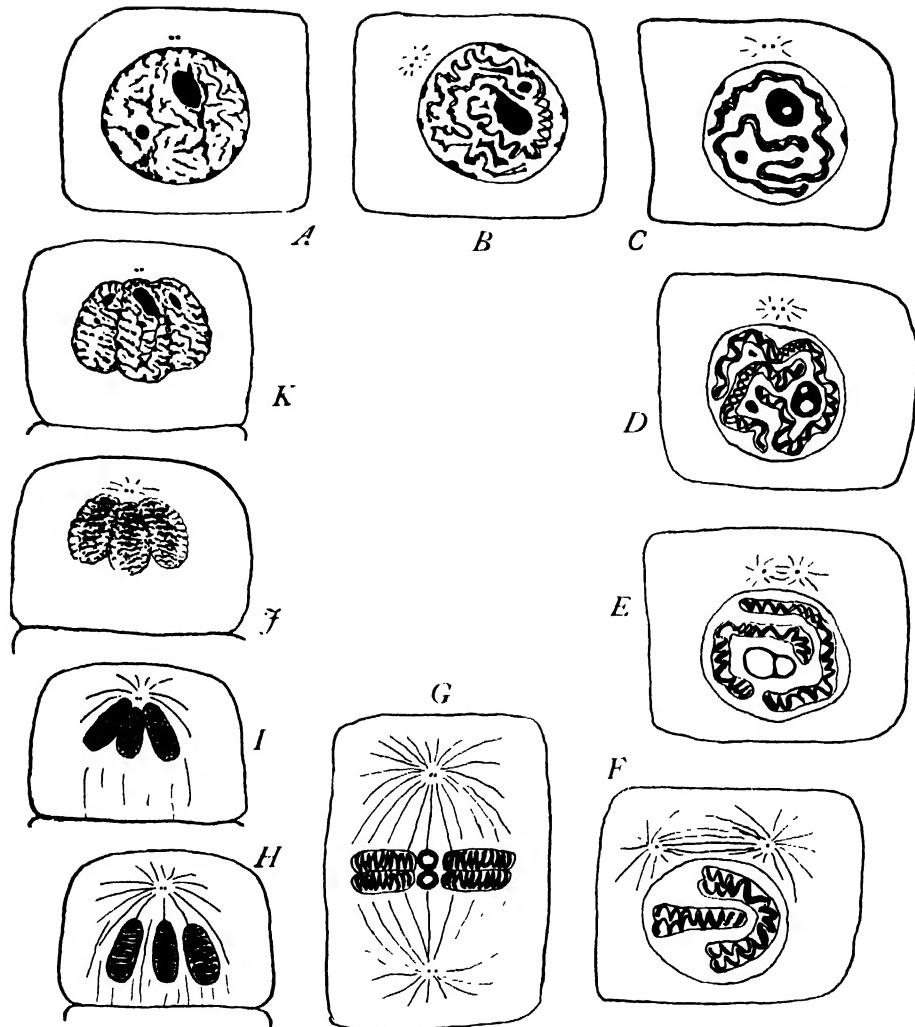
Figure 4.— Diagrammatic representation of the chromosome cycle in cell division, following *Ascaris megalocephala* quite closely. A. *Interphase stage.* The gene-string is denuded of chromatin and is not seen. Shows basichromatic net-knots and the beginnings of oxychromatic plasmosomes B. *Early prophase.* Gene-string already split; daughter strands forming basichromatic sheath and showing paired chromomeres. Loose spirals and coiling is remnant of tight coiling of previous stages. C. *Prophase, slightly later.* Beginning of oxychromatic layer between chromonemas; inception of common pellicle. Beginning of secondary double spiral Basichromatic net-knots gone, oxychromatic plasmosomes becoming achromatic. D. *Prophase.* Common pellicle advanced; shortening of double spiral, with formation of new loops and coils; old loops and coils nearly straightened out. E. *Late prophase.* Chromosomes much shorter; plasmosome nearly achromatic. F. *Early metaphase.* Double coil unraveling into two. G. *Metaphase, side view.* Middle chromosome in end view, showing basichromatic chromonema tightly coiled peripherally, oxychromatic core, and pellicle. H. *Anaphase.* I. *Late anaphase.* Gene-strings beginning to shed basichromatin. J. *Telophase* Basichromatin shed and beginning to vacuolate and change to oxychromatin; chromosome pellicles distended and fusing. K. *Late telophase.* Nucleus reformed by fusion of chromosome pellicles. Basichromatin net-knots; beginning of oxychromatic plasmosomes. Gene-strings denuded of basichromatin by deflocculation and therefore invisible.

have attracted much study as yet. Accordingly, recourse was generally had to material that was killed (fixed) and then stained with various dyes which stain the chromosomes while leaving the nuclear matrix relatively uncolored. But observations on this fixed and stained material have enabled the disposition of the gene-strings within the nuclei and the chromosomes to be arrived at with a high degree of probability.

In the following account, the characteristics which the genetical facts force us to attribute to the gene-string, are used in the interpretation of the cytological observations. The genetical findings are more reliable and more delicate in determining the finer details of chromosome structure and behavior than are the microscopical findings, which must be fitted to the genetical requirements as best we may. Owing to lack of space we shall generally restrict our account to what we regard as the most characteristic condition or relation at each phase in the cycle of chromosome reproduction. The omissions will comprise numerous variations, mostly in the relative time of onset, in the relative duration, and in the relative degree of development of the specified condition, and also alternative paths followed in well established cases. In the main, our account and illustration (Fig. 4) will adhere fairly closely to the conditions observed in *Ascaris megalocephala* (Bonnevie, 1908, 1913; Vejdovsky, 1912, 1926).

It is here assumed that the gene-string is a *filar* or nematic structure whose
• longitudinal bonding is primarily *the attractive forces exercised by the individual genes and causing adherence between adjacent genes*, rather than that the genes are secondarily held in line like beads on a string or like peas in a pod. *At most stages an envelope does secondarily exist*, through the synthesis of materials and their accumulation about the gene-string. This envelope tends to reinforce the primary bonds, and serves, by its greater bulk, to reveal the course of the gene-string concealed within it. Cytological examination shows long, tenuous threads to be present in the nucleus, especially at the close of the interphase. These threads have been clearly observed in living cells, where they are sharply separated from one another and show independent movements past one another without anastomosing. (Chambers, 1915; Chambers and Sands, 1922; Sands, 1925; Shiwago, 1926; Sakamura, 1927.)

Since the primary bonding is gene-to-gene, it is assumed that the gene-string does not change its actual length, by stretching or contraction, except to a subordinate degree. It may occupy a space shorter than its characteristic length through being thrown into loops, or into a rough corkscrew spiral. In the resting nucleus, the gene-strings, each with its secondary envelope, would tend to extend rather freely, and the fine threads seen in a very early prophase nucleus (Fig. 4B) are of this extended type. But since the length of the thread is generally greater than the diameter of the nucleus, the prophase threads are forced, by their elasticity, to lie about the periphery, where they are crowded into sinuous loops.

As will be explained in greater detail later, whenever a gene-string is carried within the pellicle of an individual condensed chromosome, it is confined to a far greater degree, and apparently exists in the form of a rough corkscrew spiral whose turns occupy the periphery of the chromosome pellicle. The gene-string, with its secondary accumulation, resembles an elastic rubber rod, or small rubber tube, and its coiling within the chromosome pellicle is illustrated by the peripheral coiling of the rubber tube when it is forced inside

a glass tube of greater diameter and shorter length. Cytological examination of condensed chromosomes in the prophases, metaphases and anaphases, quite frequently reveals this spiral structure. However, it was long overlooked, and is even now hard to demonstrate except in favorable species, since the whole contents of the condensed chromosome stain so deeply (especially with iron haemotoxylin, the most generally used stain) that internal structure may be obscured. The name *"chromonema"* was given by Vejdovsky (1912) to the coiled thread wherever it was visible within a chromosome. It must be remembered that *the chromonema is not itself the gene-string, though it may be assumed to carry the gene-string within its substance.* The relation of the gene-string to the chromonema will be considered in more detail further on.

In preparation for each chromosome division it must be assumed that there is a splitting of the gene-string into two daughter gene-strings which are precisely equivalent. This involves, or, more properly, is the result of, the separation of each gene into two daughter genes. The reproduction of each gene involves a growth of the gene material into double amount and its separation into two bodies that are qualitatively equivalent.

Each gene-string must not only split into daughter gene-strings, but these gene-strings must be completely separable from one another. The metaphase chromosome just prior to division must then contain two spirals independently coiled and lying side by side without entanglement (Fig. 4G).

The actual split of the gene-string may have occurred before the coiling and the condensation into the metaphase chromosome. It might have occurred in the previous resting nucleus where the gene-strings are free to straighten out. In many species a duality has been clearly seen to have been present at the re-emergence of the chromosomes within the resting nucleus (Fig. 4B). Wherever this "prophase split" is discernible, we may assume that *each strand is the carrier of one of the two daughter gene-strings.* Subsequent coiling of the gene-string, with the shortening of the prophase strand into the condensed form of the metaphase chromosomes, would not re-entangle the daughter gene-strings, even if, as frequently happens, the prophase strands later fuse into a seemingly simple body, the early metaphase chromosome.

The actual split in the gene-string may equally well have occurred *before the interphase,* while the gene-string was still coiled within the telophase or anaphase chromosome. In this case only the mechanical separation would occur when they straightened out in the interphase.

Moreover, the two daughter gene-strings might form a common double spiral in a single-stranded prophase chromosome, without thereby being unable to separate cleanly at metaphase. This would require only that, *in the condensation, the spiral reverse the direction of its turns by the interposition of a loop at intervals.* In that case the entanglement produced within one section of the double spiral would be compensated for in the adjacent section. Complete disentanglement and separation could then take place through slight tractions applied at the points of reversal; for the coils of the spiral are flexible and could glide through each other for a few turns without serious resistance. Such loops and reversals are common in the drawings of the chromonema by various authors.

There is another method by which secondary double spirals could be formed, of a nature such that the two sub-spirals could untangle and separate while still in the coiled condition. This would be possible if, *for each turn of the spiral, there were at the same time a twist of the two threads about*

each other in the reverse direction (Kuwada, 1926). Such a condition would arise automatically, if, during the contraction, the ends of the two threads were held relatively fixed and the coiling occurred *between* the ends. We are supposing that the reason for the coiling of the two gene-strings against their elastic stress is, in large part, the shortening of the chromosome pellicle which contains them. The pressure of the pellicle against the ends, or the embedding of the ends in the pellicle would fulfil this condition. In view of the many observations, that show the early metaphase spiral as apparently single, or with only partial splitting into two threads, we are inclined to hold that in certain species or at certain stages, a secondary double coiling is an actual fact, and that the disentanglement comes through the inclusion of loops with compensating reversals in the direction of the turns, or through the inclusion of a compensating twist of the threads for each turn, or, more probably still, to a combination of both these methods.

It is not impossible mechanically, though far less probable in point of fact, that the split might occur after the parental gene-string was coiled in the prophase chromosome as a single spiral. In this case an outside bipolar force would be needed to orient individually each dividing bipartite gene in a particular direction. It is a matter of observation that exactly at this time a strong bipolar force exists, and that the chromosomes are drawn into the axis between its two poles and there are oriented "edgewise" to the poles. The two "edges" of the metaphase chromosome represent the two daughter chromosomes about to separate. It is also reasonable to suppose that the diastral spindle orients a metaphase chromosome into this position through pulls individually upon the genes of the two strings. The bipartite appearance of a metaphase chromosome might then represent the net effect of the forced orientation of the successive genes, as division of each occurred. The plane of separation, at right angles to the spindle axis, would travel along each spiral, displacing one daughter gene toward one pole and its sister to the other, in sequence for the genes.

A split of the gene-string occurring close to the point of separation of the chromosomes carrying the daughter gene-strings, is the least probable situation. We should suppose that the split in the gene-string would normally occur *at least as far back as the preceding anaphase.* For *Tradescantia* and *Podophyllum,* Kaufmann (1926 a, and 1926 b) holds that a split is already visible in *the late prophase of the preceding division, more than one complete cell generation removed.* It is also to be remarked that the actual split in the gene-string must have occurred some time before the products of the split have reached a visible distinction through the accumulation of synthesized material about each.

THE RELATION OF THE GENE-STRING TO THE CHROMATIN AND TO THE CHROMONEMA

We may now undertake a more detailed consideration of the relation of the gene-string to the chromonema, to the visible chromosome, and especially to the materials within and about the chromosome.

In many species, painstaking examination has been made of the internal structure of chromosomes. The study has been predominantly on fixed and stained material. The most favorable stage for examination has been held to be when the chromosomes are longest and thinnest. This is at the time of

re-emergence of the chromosomes within the nuclear membrane at the close of the interphase stage (Fig. 4B). Especially favorable in some respects have been the early prophases of the maturation divisions—that is, of the cells that are about to give rise to the gametes.

It will be recalled (*vide supra*) that an interphase nucleus is formed by the fusion of the telophase chromosomes, whose pellicles become the common interphase pellicle, whose accumulations of semi-fluid colloid become the sap of the interphase nucleus, and whose cores, the gene-strings, are delivered as coiled threads to the interior of the nucleus. The gene-strings are themselves invisible, and even the chromonemas may and generally do become invisible (unstained) and remain so during the resting phase of the nucleus. When visible chromosomes re-emerge, at the beginning of the prophase stage, they are very slender, long threads, quite sharply stainable. In position, each thread may still occupy the place of a telophase chromosome, but usually there has been considerable straightening of the coils into sinuous loops and also some migration of the thread through the sap toward the periphery of the nucleus. The prophase threads, especially easy to see in the maturation prophases, are usually not homogeneous, but show a series of granules (Fig. 4B). These granules, the *"chromomeres,"* may differ among themselves by having specific relative sizes, and they maintain definite positions and seriation along the thread. While the differentiation of the chromomeres speaks strongly for a qualitative difference along the thread, they must not be identified with ⸱ genes. *The total number of such visible granules is far smaller than the total number of genes that must be supposed to occupy the chromosomes.*

However, as will be seen later in detail, the genes are seats of exceedingly active catalytic activity, and it is here suggested that *the chromomere granules are local accumulations of freshly synthesized materials. The specific relative size of a particular granule would be an index of the relative activity of the synthetic and accumulative processes carried out by a gene at its core.* The average activity in the production of accumulative stainable materials is represented by the nearly homogeneous lightly staining thread connecting the larger and more strongly staining chromomeres. This thread may be supposed to carry the gene-string, and to be made visible by the accumulations of synthesized stainable materials about each gene, the separate accumulations being indistinguishable on account of their small volumes.

Somewhat later than this stage, the local differences have partly disappeared and the whole chromosome, as stained, is a coarser thread and still less coiled. We may assume that this coarser thread represents the same gene-string surrounded by, or embedded in, *a greater accumulation of synthesized materials. The sheath of the coarser chromosome may be assumed to come from a growth and a fusion of the accumulations about the individual genes.* As these substances are synthesized, the molecules are held together in a growing mass by cohesive forces which inhibit the disruptive action of the thermal agitation of the molecules. The materials synthesized are different in physical and chemical characteristics from the surrounding nuclear sap. Accordingly, the peripheral molecules of each mass become oriented in contact with the surrounding milieu, to form an interfacial film. Secondarily, this film is increased in definitiveness, partly by the precipitation of products due to interaction between peripheral materials of the mass and the surrounding materials of the nuclear sap, and partly by absorption and orientation of still ⸱ other materials. As the accumulation about each individual gene grows by the

continual synthesis, and by the penetration of water and other permeable substances through the pellicle just formed, *these pellicles are carried outward into mutual contact lineally;* whereupon, the interfacial pellicles are modified in character or *disintegrated.* The modification would be least, but the disintegration greatest and most rapid, where the synthesized materials on the two sides of such a double membrane are most alike in composition. Staining reactions and chemical tests (not very delicate) suggest that there is a high similarity in at least some products of these syntheses. Probably the material most universally present and greatest in amount, is what has been called *"basichromatin."* Whether this is one substance or a class of closely similar substances is here immaterial. It is this material that is stained by basic dyes such as methylene green, and establishes the visibility of the chromosomes in the usual cytological preparation. Thus, with the accumulation of the specific synthesized products, including basichromatin which is common to all, *the pellicles between adjacent accumulations break down, while the portion of each in contact with the general karyoplasm is maintained, and forms a pellicle common to the whole string of genes.* It must not be forgotten that microscopic dimensions are still enormous as compared with molecular dimensions, and it should not be surprising that the accumulations about the genes should merge into a common store at so early a stage that chromosomal threads, even though still of small diameter, may appear of uniform section over considerable lengths.

The coarser threads, just described, undergo further growth in diameter, and a shortening with a further decrease in the coiling or sinuosity. This is probably the most interesting stage in the cycle of the chromosome condensation, for it involves the *differentiation of the coarse thread into a chromosome carrying a coiled chromonema* (Fig. 4C). The growth in diameter of the coarser thread is by further accumulation of synthesized materials, with probably an increased diffusion of water and solutes through the pellicle, which is thereby distended. Furthermore, it is known that the substance basichromatin, is very sensitive to hydrolysis, and that there exist, in the nucleus, chromatin-hydrolyzing enzymes, the nucleases (see general review by A. P. Mathews, 1924). We may now *assume a progressive hydrolysis of the basichromatin into a new substance,* which we identify with the *"oxychromatin,"* of the usual cytological nomenclature. That oxychromatin is derived from basichromatin by some sort of transformation is more certain than that the mechanism of transformation is hydrolysis, as suggested here. Oxychromatin does not stain with basic dyes, but instead stains with eosin and other acidic dyes. The most generally used nuclear stain, iron haemotoxlin, and likewise safranin, stain *both* oxy- and basi-chromatin and are nearly useless in distinguishing between them. The transformation will produce a rapid change in the proportion of basichromatin to oxychromatin in that part of the common accumulation *farthest from the point of synthesis.* Just beneath the pellicle there is continual transformation of basichromatin into oxychromatin, while the supply of basichromatin there is maintained only through the outward migration of molecules from the synthetic surface of the gene-string. Thus, *a new layer becomes interposed between the pellicle and the gene-string* with its direct accumulation of basichromatic materials. The pellicle maintains its continuity, but must change its constitution since it is now at the interface between the nuclear sap and the peripheral colloid, instead of between the nuclear sap and the direct accumulations. A highly characteristic property of basichromatin is its tend-

ency to form *stiff gels* (Mathews, 1924, p. 80). The gene-string and its immediate envelope of gel thus become suspended like *an elastic rod in a fluid colloid within a pellicle*. The fluid contents and the pellicle must tend strongly to round up, because of surface tension, changing from the greatly extended surface characteristic of a narrow cylinder toward the minimum surface of a sphere. The elastic resistance of the gene-string and its envelope of gel successfully opposed this tendency to contraction, so long as the pellicle was simply the outer surface of the basichromatic gel. With the interposition of a fluid, the contraction due to surface tension would have only to overcome the elastic resistance to coiling of the thread. The thread would first be forced into a sinuous zig-zag within the narrow diameter of the pellicle (Fig. 4C), but as shortening and concomitant widening of the space within the pellicle occurred progressively, loops and then coils would appear (Fig. 4D), and the thread would become disposed as a rough spiral within the periphery of the chromosome pellicle. Thus the whole chromosome finally becomes a short cylinder or sphere (Fig. 4D, E, F, and G).

It is probable that surface tension is not the only force at work to produce the coiling. It is well known that chromosomes tend to lie well separated from one another as though they were mutually repelling one another (see especially R. S. Lillie, 1901, 1903). But since the space within which they lie is limited, this repulsion would tend to compress each chromosome.

The pellicle of the chromosome, at the interface between the oxychromatic phase and the exterior phase, the nuclear sap, is probably itself a third colloidal phase (Seifriz, 1921), and as such, is subject to ageing phenomena, with *syneresis* or *shrinkage*. A familiar example of syneresis is the contraction of a blood clot (Graham, 1864). The syneretic contraction of the pellicle would act in the same direction as surface tension, and would force the chromonema to coil within the more fluid phase oxychromatic. Moreover, the colloidal oxychromatic contents of the pellicle are only semi-fluid; they really form a weak gel, which is also subject to syneresis. In the formation of the oxychromatic phase, the semi-fluid would tend to aggregate first within the concavities of the loose turns and loops of the prophase chromonema (Fig. 4C), and in its shortening, pull these original loops closer together (Fig. 4D).

Internal forces, like those in the coiling of a tendril, may be a large factor in the coiling of the chromonema. The coiling of a tendril is an especially close analogy, since, when the tip of the tendril has secured a hold about a twig, the coil then forms between the two ends which are kept from rotating.

At this stage, the late prophase, staining shows the chromosome body to possess a sharp boundary or pellicle, a basichromatic chromonema, and a relatively clear space within the spiral and between its adjacent turns. (See Fig. F, reproduced from Vejdovsky, 1912, in E. B. Wilson's book, "The Cell," p. 137.) Careful differentiation is required, since the fluid colloid zone still contains a considerable proportion of unhydrolyzed basichromatin. Examination of the living late prophase chromosomes (Chambers and Sands, 1922) shows the structure to be an axial sol and a cortical gel. This cortical gel is to be interpreted as the closely appressed turns of the peripheral spiral. Barenetsky, who gave the first description of spiral structure in chromosomes (Barenetsky, 1880), *clearly saw the spiral in living late prophase chromosomes*.

As we have seen above, the chromonema at early metaphase (Fig. 4F) is to be interpreted as composed of two sister gene-strings coiled together secondarily within a common basichromatic gel (see Fig. 119, Vejdovsky, 1926).

The release of the sister coils from each other would be accomplished by a gel-sol transformation * of the common basichromatic envelope—a kind of deflocculation that would spread more basichromatin throughout the pellicle and further obscure the cytological picture. The released sister spirals (Fig. 4F) could then ravel out into two spirals lying closely side by side in the common pellicle (see Fig. 85, Vejdovsky, 1926). The metaphase chromosome might usually present the appearance of paired strings of chromomeres, since the paired turns of the spirals would be obscured by the masses of basichromatin and simulate paired beads (Kuwada, 1926). In the division of the metaphase chromosome, these spirals are drawn apart from one another, with a longitudinal splitting of the pellicle. The split is observed to begin as a notch at the free end of chromosomes, that is, at the end distal to the spindle-fiber attachment. The notch deepens to a cleft and develops into a split that travels along the chromosome toward the spindle-fiber. The freed chromosomes are then drawn apart and to the poles along the lines of force shown by the diastral spindle (Fig. 4H).

A different aspect of basichromatin begins to appear in the late telophase chromosomes (Fig. 4J). By this time a considerable synthesis of basichromatin has occurred. With some stains, it is seen that vacuoles begin to appear in it, which are here interpreted as centers of hydrolysis of basichromatin into unstained oxychromatin. Considerable swelling accompanies the hydrolysis. As the hydrolysis proceeds, the vacuoles become larger and confluent, so that the remaining basichromatin is a reticulum very irregular in disposition. This process is perhaps most clearly seen in certain species in which the telophase chromosomes remain separated and distinct for a considerable period, during which each is resolved into a vesicle like an interphase nucleus, but smaller, since it represents only a single chromosome. In each of these "karyomeres" the remnants of the eliminated basichromatin appear as shreds, "net-knots," loose strands, and irregular lumps. The disposition of basichromatin at this stage has little relation to the chromonema, which is so strongly denuded of basichromatin by deflocculation as to be invisible. The remnants are perhaps more likely to show where the chromonema is *not* present, for they may collect in the spaces around and between the turns of the gene-string and form a ragged peripheral spiral.

Still less reliable is chromatin as an index of chromosome structure in the interphase, where, in addition to the vanishing fragments of basichromatin, there is a secondary aggregation into rounded lumps or droplets principally oxychromatic in content. These "nucleoli," or *plasmosomes*, are a conspicuous feature of interphase and prophase nuclei, but disappear during prophase, often by the appearance and growth of vacuoles within them (Fig. 4C, D and E). This is evidently a fresh onset of hydrolysis into a material different from oxychromatin, but quite similar to the nuclear matrix, and relatively *achromatic*. There is no genetic continuity of basichromatin from the anaphase to the prophase chromosomes. The basichromatin of the anaphase chromosomes is shed, and this eliminated chromatin, after lingering as shreds or transforming into oxychromatic droplets, is gradually hydrolyzed out of existence as chromatin. *When the prophase chromosomes appear, the basichromatin is a new formation sharply confined to a definite thread without relation to the remnants of anaphasic basichromatin.* (Compare Figs. 419I and 419J, after Richards, 1917, in E. B. Wilson, "The Cell," p. 894.)

* See paper by S. C. Bradford on "The Sol-Gel Transformation" in Vol. 1 of this series.

There has been a strong tendency to regard the nucleoli as a reserve of basichromatin which is used in the formation of the prophase chromosomes. Our assumption is that the catalytic surfaces of the genes within the prophase chromosomes, are the source of the basichromatin of the prophase chromosomes. The concentration of basichromatin within the pellicle of the prophase chromosome would be far higher than in the surrounding nuclear plasm where the basichromatin has largely hydrolyzed into oxychromatin, or still further, into achromatin. Any diffusion of basichromatin would follow the concentration gradient from within outward. It is obvious that if diffusion occurred in the reverse direction, the increasing concentration of basichromatin would, according to the law of mass action, tend to inhibit the catalytic synthesis of basichromatin at the gene surface. The *materials* for the synthesis of basichromatin must diffuse inward, but these are probably relatively simple substances. Since the chromatin is relatively very rich in phosphorus, there may be a phosphorus cycle within the nucleus, the basichromatin of the nucleoli hydrolyzing to oxychromatin and ultimately to a phosphorus compound that can diffuse back to the catalytic surface. But at each cell division in growing tissue two daughter cells are formed, each with a phosphorus demand equal to that of the parental cell. Accordingly, the internal phosphorus cycle can be of only temporary importance, while diffusion from the exterior is essential.

In diffusing outwardly from the gene surface, synthesized materials may pass through several zones which alternate with interfacial membranes. For example, at the late prophase there are: first, the zone of basichromatic gel of the chromonema with its surface film; second, the largely oxychromatic sol of the chromosome with its pellicle; third, the nuclear sap with its nuclear membrane, and, fourth, the cytoplasmic colloid with the plasma membrane and cell wall. But materials can pass outward by a different means than simple diffusion, and can arrive at the plasma membrane without traversing any pellicle. This is because there is a genetic continuity between the inner pellicles in series, and accordingly the contents of one pellicle are automatically and passively discharged into the next outer pellicle in succession, at characteristic points in the cycle of cell reproduction. At the very earliest stage following gene division, each gene begins to accumulate its individual products. These primary accumulations grow, and their primary boundaries or incipient pellicles fuse to form a secondary pellicle, the gene-string pellicle, with delivery of the primary materials into the secondary zone. During prophase the chromonema pellicle becomes, by delamination, the chromosome pellicle, and this tertiary zone receives the secondary materials directly. At the telophase the chromosome pellicles fuse to become the nuclear membrane, with reception of the materials from the tertiary zone. Finally, at metaphase, this quartenary membrane disintegrates, delivering the materials of the fourth zone into the cytoplasm. By a four-dimensional path, one of whose coordinates is time, materials that were once inside the inmost of a succession of concentric pellicles, are now outside, without having diffused through any of them.

CHROMATIN ELIMINATION

The phenomenon of "chromatin diminution" from metaphase chromosomes, is probably best explained in terms of an *excess* accumulation between the chromonema and the chromosome pellicle. Thus, in certain lepidoptera the chromosomes of the ordinary divisions have a characteristic size. The chromo-

somes of the primary oöcyte, however, are increased to a *larger size* during the long growth period. We may assume that this "over growth" is the result of syntheses continued within the chromosome pellicle during the extended growth period. But after the actual separation of the chromosomes, the daughter chromosomes are again small and the excess material is left behind in the equatorial plate, that is, in the area which the metaphase plate occupied. Staining reactions of the enlarged chromosomes show a considerable *peripheral zone* that is weakly basichromatic and weakly oxychromatic, so weakly basichromatic that shortly after it is "eliminated" and thereby loses its connection with the source of basichromatin, it stains purely as *oxychromatin*. (L. C. Fogg, unpublished.) It may be surmised that the old pellicle and the excess accumulation of semifluid oxychromatin is scraped off by the stiff gel of the astral figure, as the chromosomes start to traverse the more liquid rays. Thus, "chromatin elimination" from metaphase chromosomes is quite simply that, *and is in no sense a loss of the hereditary units from the chromosomes.* Only in the stage at which it occurs does this elimination differ from the general elimination of chromatin at the telophase and interphase, in the manner already described above.

THE SIGNIFICANCE OF CHROMATIN

That chromatin, particularly basichromatin, is the substance of greatest importance in the nucleus and is the material concerned with heredity, is a view that is widespread in the literature of cytology of heredity and of biological chemistry. This view seems to us entirely incorrect. *Probably chromatin is invariably a secondary synthesized substance, one of the formed materials of the cell.* It is not to be regarded as genic substance nor even as self-reproducing. If it plays a rôle in development, it is as a messenger from the genes and not as a primary directive agent. Its real significance probably lies in connection with the nature of genes: Seemingly basichromatin is universally produced by genes, which implies that *a particular process, and hence a particular structure is common to all.* The genic molecules are conceivably all modifications of one basic pattern on which depends the autocatalytic property, and the specific heterocatalytic syntheses (*vide infra*) that control heredity are then due to special modifications of, or additions to, this basic structure. The most fundamental property of the genic molecules is that of their autocatalytic growth and reproduction, and it is possible that basichromatin is a by-product of that universal activity. Thus, the Y-chromosome of Drosophila melanogaster, and of various other animals, seems to be entirely normal in the amount of chromatin but relatively deficient in genic action. It may be surmised that the genes of the Y-chromosomes have mostly lost the special modifications which control the heterocatalytic syntheses but retain the basic structure responsible for their reproductive autocatalysis. They are "stripped" genes.

THE REPRODUCTION OF THE GENE

The reproduction of the gene and its evolution by mutative changes may now be considered in detail. In describing genic reproduction, the present account is a modification of the earlier application of the idea of autocatalysis to genic phenomena (Bridges, 1922, 1923; Muller, 1922), which was itself

inspired by Troland's general application of autocatalysis to the origin of life and the reproduction of living material. (Troland, 1914, 1916, 1917.)

The facts of linkage have led to the conception of the gene as a definitely delimited, definitely localized sub-unit in the structure of the core, or gene-string, of the chromosome. To this must be added the view that each gene is a definitely organized self-reproductive unit. With each cell generation, the original gene gives rise to two daughter genes identically located and with the identical characteristics of the parental gene, as judged by the identity of effects in successive generations. In the increase in the amount of the substance of the particular gene, preparatory to division, constituents that were present in the surrounding medium interact or condense to produce a specific end-product. In the case of contiguous but unlike genes, the raw materials present in the surrounding medium are essentially the same. The primary difference between the two types of synthesis carried on simultaneously and in close juxtaposition in the cell, must be sought, not in the cell plasma at large, but in the particular definite locus in which each synthesis occurs and continually reoccurs. Effective synthesis of each type is restricted to a particular locus, and hence must depend upon the action of some material situated at that particular locus. But when this synthesis has occurred, the substance has not been used up, or else it has been reconstituted, for in the daughter loci the same reaction is repeated at the succeeding growth period. This suggests that the condensation is catalytic, and specifically catalytic, since in adjacent loci different condensations occur. The situation can be accounted for on the assumption that the specific catalyst is the genic material itself. This assumption is essentially the chemical conception of *autocatalysis,* explained in some detail in Part I of this paper. The reaction may be called autocatalytic if the genic material is both catalyst and an end-product. The characteristic molecules of each gene are assumed to be such, that, if the proper raw materials are present in the surrounding medium, interaction occurs and one of the reaction products is the initial type of genic material, which thus increases in amount. The autocatalytic synthesis of a gene may be formulated: $G + C_G \rightarrow 2 G + P_G$, in which C_G represents the totality of raw materials taken from the medium in the synthesis, and P_G represents the series of end-products that are returned to the medium. The two daughter genes $(2 G)$ separate from each other, and each retains its definite position in one of the two daughter gene-strings.

If, as seems certain, the genic molecules act also as heterocatalytic enzymes, other specific end-products of greater variety are synthesized. Considered from the standpoint of the end-products, genes are chemical factories, each of which is synthesizing a characteristic set of end-products which are delivered ultimately to the common cytoplasm. The various products influence the constitution of the nucleoplasm and of the cytoplasm directly by being constituent parts, and indirectly, by their interactions with one another and with the host of materials diffusing in from the milieu of the cell. Also the products of one gene may act as the raw materials for the syntheses carried out by other genes. In producing and controlling development, the genes of the entire complement act together, since all are liberating their chemical products into the common cell plasma. But since the products of the different genes are different, they take effect in different ways upon the developing organism. Some of the genes will have much effect upon one character and only slight effect upon another. Each character will be determined by all

the genes, but in each case most of the effect will be produced by a relatively small proportion. For example, in Drosophila the products of one gene are predominantly concerned with eye-color, but have accessory effects upon the wings, while the products of another gene influence greatly the number of bristles, but have also slight effects upon a dozen other characteristics.

Mutative Change of the Gene

In dealing with Mendelian allelomorphs (i.e., alternative characters), the evidence from linkage proves that the two contrasting effects are due to materials having the same location in the gene-string. New allelomorphs are frequently arising in the course of the observations on Drosophila, and each of these must involve the extinction of the specific gene that formerly occupied that locus. The extinction may be, and usually is, by the modification of the old gene into a new gene which thereafter occupies the same place. The production of the old type of genic material has ceased and in its place a new type is being produced. The change in the synthesis of genic material may be formulated: $G_1 \ldots \ldots G_1 \rightarrow G_2 \ldots \ldots G_2$. The details of this transforming genic mutation ($G_1 \rightarrow G_2$) will be examined later; in principle it is by change of genic components or sub-units. The autocatalytic reaction $G_2 + C_{G2} \rightarrow 2 G_2 + P_{G2}$ (together with whatever heterocatalytic action is present) liberates a set of end-products (P_{G2}) that is different from that characteristic of G_1. Accordingly, the effect of G_2 upon the characteristics of the developing organism is different from that produced by G_1.

Chromosomes as Aggregates of Genes. Genic Balance

It was mentioned before that the character changes observed when a particular chromosome is present in unusual number, are interpretable in terms of sub-units of the chromosomes, namely, the genes. This method of formulation, called *"genic balance,"* was used to account for the character changes due to losses or gains of sections of chromosomes (sectional deficiencies and sectional duplications), as well as for the character changes due to duplication or deficiency of whole chromosomes (Bridges, '21, '22, '23, '25).

The central idea of genic balance is that, in producing development, *the genes of the entire complement act together, but some of these genes are acting to make a given character more pronounced, while others are tending to restrict its development.* The grade realized by the character is that corresponding to the net effect of these opposing "plus" and "minus" modifiers. The directions of modification for each character are in general numerous and diverse, and it is for the sake of simplicity of statement that they are lumped together as "plus and minus" modifiers, without specification as to the type of effect. For example, the trident pattern on the thorax of Drosophila may become lighter or darker in intensity, blacker or yellower in color, sharper or more diffuse, larger or smaller, and it may change in shape by changes in any of its regions. *A change in the effectiveness of any of the modifiers may occur with genic mutation.* In mutant races the point of equilibrium has been shifted by the transformation of a modifier of standard effectiveness into a modifier of a different grade, with a corresponding shift in the grade of the character. The "normal" point of balance for each character is that which exists in the wild-type or standard material. A lesser development than that shown by the

wild-type is due to a "minus" modifier of the character in question; for example, the gene for "miniature wing" is a minus modifier of wing length.

Linkage experiments show that the various kinds of genes are distributed pretty much at random among the different chromosomes and along each chromosome. But since the number of genes with a given tendency is relatively small, any particular chromosome or limited section of chromosome is unlikely to contain these genes in the same proportion as they exist in the entire complement, and still less would it be likely to contain the normal proportion of every kind. The loss of a particular chromosome, or section of a chromosome, would ordinarily remove more minus than plus modifiers (or vice-versa), and since in that case more plus than minus modifiers would remain in action, the grade of the corresponding character would be shifted in a plus direction. Thus, a deficiency for a chromosome, or a section of a chromosome, may cause the appearance of a whole complex of character changes. The complex of altered characters would be inherited as a dominant, that is, would appear in all offspring that failed to receive from the parent the chromosome in question.

As an example of such effects we may use the changes caused by the loss of one of the small round "fourth-chromosomes" of *Drosophila* (Bridges, 1921). Individuals having only one fourth chromosome, all the other chromosomes being normally present in duplicate, show changes in many characters, among which may be mentioned smaller size, smaller bristles, later hatching, poorer viability, paler body-color, darker trident pattern, larger roughish eyes, and shorter blunter wings. Each of these differences corresponds to a character *for which the fourth chromosome is internally unbalanced*, that is, for which the ratio of plus to minus modifiers is different from that of the whole complement. For all the characters in which there is an internal preponderance of plus modifiers, the grade is shifted in a minus direction by a loss of one fourth chromosome, for example the shorter wings and paler body-color.

It was anticipated that *the characters of an individual having an extra fourth chromosome would be shifted in the opposite directions from those shown by the haplo-II individuals* just described. In certain cultures genetical results showed that triplo-IV individuals must be present, but nevertheless they could not be identified until a deliberate search was made for flies with characters the opposite of those already known for haplo-IV. They were presently found through their smaller, smoother eyes, longer, narrower wings, darker body-color, paler trident pattern, etc. (Bridges, 1923). However, while each character change had the opposite sign to that of the haplo-IV, the amount of change was relatively slight for each character. On the other hand, loss of both fourth chromosomes makes such great character changes that the nullo-IV individual cannot live beyond the egg stage.

A whole series of sexes have been found for *Drosophila* (superfemales, females, intersexes, males, supermales) and have been shown to be *due to changes in the ratio of X-chromosomes to autosomes*, the X-chromosomes being internally unbalanced with an excess of female-tendency genes, and the autosomes being internally unbalanced with an excess of male-determining genes (Bridges, 1922, 1925).

THE INTERNAL STRUCTURE OF GENES. THE GENELS

We may now pass on to the interpretation of the properties, the reproduction and the evolution of genes in terms of the internal structure of genes.

Through his analysis of hereditary effects, the geneticist has possibly been able to penetrate one stage below the gene, to sub-units called "gene elements," or, as we propose to call them, *"genels."* This tentative advance was accomplished through study of variegated characters and of genetically unstable characters. The basic assumption was made by E. G. Anderson (unpublished by him) that the genetical behavior of the fine red-and-white striping seen on kernels of corn worked with by Emerson (1917) furnished evidence of *the existence within the gene of sub-units* for red and for white. Presumably one of these kinds of gene elements, let us say the white, represented the original type and the other, the red, represented the mutant type of genel. The genetic mutation may have occurred only once in the ancestry of this striped corn. Thereafter, the original (white?) and the mutant (red?) genels both existed within the gene and reproduced independently but at about the same rate. Fluctuations in the ratio of red and white genels would occur if there were no machinery to insure an exact representation of each genel in the two daughter genes. Or this machinery might sometimes miscarry, as does the machinery which normally provides a representative of each chromosome in the daughter nuclei, but which occasionally fails (non-disjunction, chromosomal elimination, etc.).

In some strains of the variegated corn, especially that worked with by Eyster (1924) the kernels have a generally diffused "orange" color as a background for the variegation. In intensity the orange varies, in different plants or strains, toward an almost colorless type or to a deep orange-red. It may be supposed that the depth of color is a rough index of the proportion of red genels to colorless genels in the gene. On this orange background appear stripes of red and white, in pairs, a red and a white stripe of equal width lying side by side and replacing the general orange background. This pairing of the stripes furnished convincing evidence that *cell-division is involved in the incidence of the striping,* and led to the conclusion that at some division of the mixed gene one daughter gene received more than the usual proportion of the red genels and the other daughter gene received a correspondingly greater proportion of the colorless genels (genelic non-disjunction). In the tissue descended from the cell to which the greater proportion of red genels was segregated, the color is a dark stripe (probably red if the ratio of red to white is above a critical value). Correspondingly, in the tissue descended from the sister cell in which the proportion of white genels is low, the stripe is light in color (or white, if the proportion falls below a critical ratio).

The visible variegation is that due to genelic non-disjunction in somatic tissue, but the differential segregation may occur also in the germinal tissue, and result in individuals and strains with greater or lower intensity of red in the somatic tissue. If the intragenic segregation becomes complete, then, on the one hand, a pure-breeding white corn results, or, at the other extreme, a pure-breeding red corn. But all mixtures, even if the minority type of genel is only a small proportion of the total genic content, would, by the same process of sorting out, continue to give red stripes or otherwise light corn and light stripes on otherwise dark-red corn.

Not all variegation can be attributed to this type of phenomenon. Eyster (1925) has used it in explaining another variegation in corn, the "mosaic" type. Demerec has found in *Drosophila virilis* three mutant genes each of which reverts to the wild type with high frequency; and two of them change over

into stable, non-reverting genes. There is considerable discussion, behind the scenes in genetical circles, as to how great a prevalence will be discovered of this instability of genes, resulting from differences in component genels. A further question is, what proportion of genes that are now stable, were unstable at their first appearance, and have attained stability through descent from a gene that had become homogenelic for the mutant genel. The frequency of unstable genes in *Drosophila melanogaster* seems to be not as high as in *Drosophila virilis*. The general point of view—the existence of genels—seems to be gaining adherents. Not all genes need be thus compound, nor indeed genes of every species.

The Line of Demarcation between Living and Non-living Units

The present, though insecurely reached, null-point of the geneticist is the genel, as the electron is the present null-point of the physicist. But our general theory of the relation between structure and properties forces us to assume that the properties of the genel are due to the properties of components of the genel. In volume the genel is a fraction of that of the gene, which, as we have already seen, has a maximal size in the neighborhood of 70 to 20 mμ. Even if we assume that the gene is composed of relatively few genels, the size of a genel is quite small. The structural analysis of the material of the genel, if it were carried through successive orders of complexity, would presently arrive at a volume corresponding to a physical unit smaller than most molecules, in fact, to the atom of the physicist.

Our thesis is that *in passing from the order of complexity of the gene or genel to that of the contained atoms we pass out of the range of life, into the range of the non-living* at some definite order of complexity. The line of demarcation which we draw is that between the molecule and the atom. The possibility of life exists for unitary masses of the order of complexity of molecules, but not that of atoms. We have already stated the criterion whereby the line of demarcation can be drawn so sharply. It is in the characteristic method of *increase in numbers* of examples of the particulate unit specified. In non-living units it is by *repeated production*, each example of the specified unit being a discrete event, discontinuous in the space-time continuum from the other examples. In living units it is by *continual reproduction*, each example of the specified unit being continuous in time with previously existing examples as a member of a chain or sequence of events *and* continuous in space as a subdivision of a periphery that included a preexisting example.

The lowest order of complexity possible for a living unit is that in which a reproductive subdivision occurs which is a direct separation into two identical units and is not an indirect result of the previous reproductive division of discrete component sub-units.

This criterion excludes atoms from the range of the living, since, for example, separately existing atoms of the specified kind "nitrogen" do not characteristically increase in numbers by the assemblage of the components of nitrogen within the boundary of a preexisting atom of nitrogen, with expansion of the nitrogen boundary, rearrangement of the internal structure, and subdivision of the contents into two nitrogen atoms. Nor is the increase in number of nitrogen atoms characteristically through the increase, within the interior of a previously existing nitrogen atom, in the numbers of a sub-

unit, each of which then forces the assemblage of components of nitrogen into a nitrogen atom and no other.

THE REPRODUCTION OF GENELS

In the description of the synthesis of the specific material of a gene, the formulation was $G + G_G \rightarrow 2\,G + P_G$, which we saw may be described as a *specific autocatalytic synthesis;* specific catalytic, since a heterogeneous mixture of materials, the nucleoplasm, gives rise to this specific material only in a specific location; autocatalytic, since this location is restricted to that already occupied by this same material, which is both catalyst and end-product. If genels exist, and the evidence seems to us strong enough to accept their existence provisionally for certain genes, then the synthesis within such compound genes can be shown to be still more local in its nature. Within such genes the genic material exists as discrete particles, and when these differ among themselves, the amounts of synthesis of each kind are in that ratio in which the two kinds existed at the beginning of the synthesis, however that ratio may have varied through inexactness in the process of partition of the products.

GENETOMOLECULES

The limit in the restriction of synthesis to a locality already occupied by the same material, is in the molecular order of complexity; for, as we have just seen, the increase in the numbers of an atom of a given kind is not characteristically conditioned by the presence of preexisting atoms of that kind. Accordingly, the applicability of the idea of autocatalysis ceases below the molecular order of complexity. Above the molecular order of complexity the autocatalytic syntheses (for example, that of a compound gene) is probably a result of molecular autocatalysis. It is clear that the autocatalytic synthesis of molecules is still within the limit of continuity in space-time. The processes involved in molecular autocatalysis are, roughly: (a), the presence of a molecule of specific structure; (b), the local accumulation of the raw materials for a new molecule, probably by adsorption on the surface of the given molecule, which thereby extends its periphery; (c), the condensation of the raw materials according to, or their rearrangement into, the pattern of the given molecule, probably with elimination of by-products; (d), the cleavage of the bipartite molecule into two equivalent molecules, which are the same in structure and composition as the original molecule (see Fig. 3).

Here again it must be stated that no molecule should be considered a vital sub-unit in a higher aggregate unless it falls within this limiting condition of autocatalytic synthesis, but that conversely the property of autocatalysis does not insure life *in the higher aggregate.* Maintenance of existence as a vital sub-unit in an organism of the cellular order of complexity, is possible only to those molecules whose maximum rate for autocatalytic synthesis is as high as the minimum rate of division of the cells.

We propose the name "genetomolecule" for this limiting unit in the box-within-box series of vital units.

The number of orders of complexity intervening between the gene and the genetomolecule is at present conjectural. *It is not impossible, nor even*

improbable, that genels are the genetomolecules. Nor is it improbable that some (or even most) genes are unimolecular and hence genetomolecules.

LIVING AND NON-LIVING COMPONENTS OF LIVING UNITS OF HIGHER RANK

The limiting unit in the series of vital units is itself wholly composed of non-vital units. Moreover, each vital unit above it is an aggregate not only of vital units but also of non-vital components under the control of the vital units. The controlled non-vital material may constitute the bulk of the contents of the vital unit. In the case of the cell we have seen that vital units, the nucleus, the plastids and perhaps a few other elements in the cyto-plasm, constitute a small proportion of the total contents of the cell. H_2O is always and everywhere regarded as non-living, even if it is a part of a living unit of higher order.

THE BIONTIC SERIES

An electron may exist as a sub-unit of a higher physical aggregate, e.g., the atom, or it may lead an independent existence as a free individual. This brings us to the problem as to what classes of independently existing bodies should be included in the "Kingdom of living things", or are individual living organisms, "bionts".

With macroscopic objects the decision is usually easy. Probably the most typical example of a biont is a human being. But no one would refuse ad-mission to the large animal and plant forms, or, in general, to the *Metabiontia*, if we may so designate the group in which the individual is clearly an aggre-gate of cellular units. More restricted in activities, and a Rank simpler in structural organization, are the *Cytobiontia* or *Protobiontia*, in which the basis of the individual is a single cell with definite nucleus and cytoplasm. In the bacteria the organization is still simpler; and is sub-cellular, in the sense that no differentiation into nucleus and surrounding cytoplasmic zone is certain. On account of this simpler organization, which has often been compared to that of a free nucleus,[4] it may be permissible to give the bac-teria a position coordinate with that of the Metabiontia and Cytobiontia as *Bacteriobiontia*. The bacteriophage, filterable viruses, and bionts of that still simpler grade of organization and generally ultramicroscopic in size may be ranked as *Ultrabiontia*. With the Ultrabiontia the limit of known biontia is reached. Considerations, which have already been given, lead to the conclusion that the hypothetical simplest biont is of the molecular order of complexity and is therefore a member of the *Moleculobiontia*.

THE CHRONOBIONTIC SERIES

We have already mentioned several of the ways in which changes can occur to one or another of the units in the *genetic series*, with effects upon all units of which they are sub-units. There remains the problem of the *origination* of the orders of genetic units in an evolutionary or time seria-tion. This problem is so difficult of real solution that the following remarks are frankly speculative and can be accepted or rejected according to the taste of the reader.

* First suggested by Huppe, 1886   For a review of this idea, see Dobell, 1910

The *biontic series,* as well as the genetic series, shows orders of complexity and progressive aggregation, although not so clearly as the genetic series. With respect to both of these series it seems evident that a unit that is itself an aggregate of lower units, would not be as likely to arise directly from non-living matter as to arise indirectly by the aggregation of sub-units which have themselves arisen previously from non-living matter (Minchin, 1912). *It seems improbable that a cellular unit, with its successively lower orders of concentrically organized substructures, could have arisen before these sub-units had themselves arisen, in a time sequence corresponding in a general way to their seriation in order of complexity.* We should expect a cellular type to arise before a multicellular, a nuclear type before a cellular, a chromosomic type before a nuclear, a genic before a chromosomic, and, hypothetically first of all, the genetomolecular type.

It seems probable that the biontic type arose before the corresponding genetic type in each order of complexity. Certainly Metabionts arose by the aggregation of cytobionts, and there was probably an immense stretch of time between the appearance of the first Cytobiont and the first Metabiont, that is, before some Cytobiont arose with the characteristics which allowed it to become a genetic sub-unit. Similarly, free-living genes or genobionts probably existed long before certain of them aggregated to form a chromosomic type, of which they were then sub-units in the genetic series. One wonders if these free-living genes may not be already known to us as the Ultrabiontia.

THE ORIGIN OF THE CYTOPLASM

The prevalent view as to the origination of the cytoplasmic-nuclear association has been that both the nucleus and the cytoplasm are specialized regions which have been differentiated within a primitive general protoplasm. The analysis just given leads to a somewhat different view—that life began with the origination of the moleculobiont, which corresponds to the lowest order of the nuclear sub-units. But the primitive homologue of the cytoplasm came on the scene as soon as this first moleculobiont began to reproduce itself. Its autocatalytic reproduction may be formulated $M + C_M \rightarrow 2M + P_M$, in which the by-products, P_M, together with whatever heterocatalytic products were formed, constituted the primitive cytoplasm. Perhaps most of the products were lost by diffusion. Some remained as a local accumulation, if not with the first moleculobiont, then with some later one, which was derived from the first by mutation, or which originated directly from abiontic matter. With the aggregation of moleculobionts, which were thereafter genetomolecules, the zone of local accumulation might become more definite. It would become more diversified in composition as now one, now another, of the genetomolecules underwent mutation and thereafter delivered to the common zone the products characteristic of the mutant genetomolecules. Basichromatin, according to the analysis given above, is one of the substances synthesized, and it possesses such physical and chemical properties that it forms a zone of gel about the genetic units, which are its centers of synthesis. By interaction with materials diffusing in from the outside, the directly synthesized materials may be transformed into others in lengthy series or in cascade formation. The serial formation of basichromatin, oxychromatin and achromatin, is an example of this type of action. In the

fully developed cellular unit the chromonematic gel, the chromosome colloid, the nuclear sap, and the general cytoplasm are the successive zones of outward displacement of the synthesized products, and *each zone is the seat of interaction between the outgoing products and the materials diffusing inward stage by stage.* Among the most characteristic of the outgoing substances are what may be called free enzymes, and it seems probable that these also act in cascade fashion.

The main characteristic of the cytoplasm and of the earlier homologues of the cytoplasm is that they are *produced* and *not self-reproductive* material. It is true that the definitive cytoplasm is inhabited by self-reproductive units. Thus, the plastids have a tremendous influence upon the composition of the sub-vital material of the cytoplasm, but the plastids may be looked upon as side-series of vital units—*Symbionts*—that have invaded the cytoplasmic zone.

E**VOLUTIONARY** S**TEMS**

Similarity of descent should result in a residue of similar features in the bionts related by descent. It is this aspect of life that is the basis of the usual classification into Phyla or streams of life (rather than into Ranks, according to the order of complexity in structure). Some clearly defined streams are the vertebrates, certain major groups of invertebrates, the dicotyledonous plants, etc. In tracing back such streams, as best we may, it is often found that several later ones may well have arisen from a single earlier one, and that the number of separate origins of life that we must ultimately assume for the higher ranks of bionts is not great, and is possibly only one. There are several facts which indicate that the genes (or genels), of a given biont, may all be modifications of one primal and basic structure.

There is a process well exemplified in modern heredity which could have been the mode of derivation of all the genes of a modern biont from a single ancestral free gene (Bridges, 1923). This is the process known as *"duplication"*, and *is essentially the addition, in a new relation, of units already present elsewhere in the old relation.* The added material is available through a failure in the normal machinery which separates the daughter units after division (non-disjunction, etc.). Thus, in a form in which each of the kinds of chromosome is represented twice $(7 + 7)$, a chromosome of a particular sort may come to exist in triplicate through a failure of the separation of the two daughter chromosomes (chromosomal non-disjunction) in the formation of an egg. This $(7 + 1)$ chromosome egg, fertilized by a normal 7-chromosome sperm cell, would give the start of a new and distinct 15-chromosome $(2n + 1)$ race. The above example is of chromosome-duplication, but group-duplication (from group non-disjunction) is very frequent. In this phenomenon all of the chromosomes of the group $(7 + 7)$, may fail to disjoin after having divided. The result is a form containing 28 chromosomes, $2 \times (7 + 7)$, and is called a "tetraploid" type. Likewise, "section-duplication" is known, in which case a piece taken from a certain place in one of the chromosomes is attached to some other chromosome, or elsewhere in the same chromosome, so that it exists in that race not only in its normal situation, but also in the novel location. Perhaps the first order of Biontia above the Moleculobiontia, was a bimoleculobiont that came through the non-disjunction of two daughter moleculobionts and their re-

attachment in tandem fashion. In any of the early stages of evolutionary aggregation, *the duplication of a sub-unit, of a unit, or a group of units, would serve to increase the biont quantitatively or give rise to aggregates of higher rank. The qualitative diversity of the biont would increase through mutations which would occur independently in the genetic units in the normal location, and also in the duplicating material.** For the units duplicated, mutation would have twice the raw material and could proceed correspondingly faster and in more directions at a given period.

SOME TURNING POINTS IN EVOLUTION

It may be supposed that the metabolic life of the early moleculobionts involved relatively few and simple syntheses. The primitive homologue of the cytoplasm was still undiversified in composition and meager in amount. The raw materials for the syntheses were predominantly in the form of isolated utilizable molecules encountered by chance. Only slowly would the concentration of organic matter and its extent of elaboration increase in the ocean. The increase would be mainly from the by-products, since the death and dissolution of a moleculobiont would probably be rare. Vast periods of time † were presumably spent in this stage, in which the Biontia might be called *chemosynthetic* since the syntheses carried out were primarily from utilization of the chemical energies derived from the chance encountered molecules, the catalytic surface being the channel of liberation and utilization of these forces.

A great advance came with the advent and development of the predatory habit. The raw materials were then secured in relatively large masses and in relatively highly elaborated forms. This constituted the beginning of the distinctively animal stem.

Another great advance (represented in Fig. 5) was the development of *photosynthesis,* or the *utilization of radiant energy in significant amounts.* This came by mutation or a series of mutations such that one of the products was the special catalytic material *chlorophyl.* This was the start of the distinctively plant stem.

The development of the power of synthesizing *cellulose* gave greater structural rigidity to the plant units but increased immobility. Possibly the fungi represent this line with the secondary loss of the photosynthetic power, or the order of development may have been first cellulose synthesis and only later chlorophyl synthesis.

It has been supposed (Minchin, 1912) that the invasion of a cellulose-producing unit by a simpler chlorophyl-producing unit gave origin to the *chlorophlast-bearing plants.*

With each advance in the synthetic powers of the plant stem, there was the possibility of a correspondingly great advance in the mobile predatory zoan units. The attainment of the *Cytobiontic* and the *Metabiontic* Rank greatly increased the powers of the unit.

In Figure 5 one stage of aggregation above that of the Metabiont is represented—namely, the colony or *Social* Rank. In the zoan stem this rank

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* Haldane and Huxley (1927) in their stimulating book "Animal Biology" have developed a similar idea of progress by successive aggregation coupled with successive increases in the powers of the bionts of each Rank.

† In Figure 5, these vast early periods are represented by the gentle slope of the beginning of the curve of rising life, and by the use of a logarithmic scale of years along the time axis and a logarithmic condensation along the axis of Ranks.
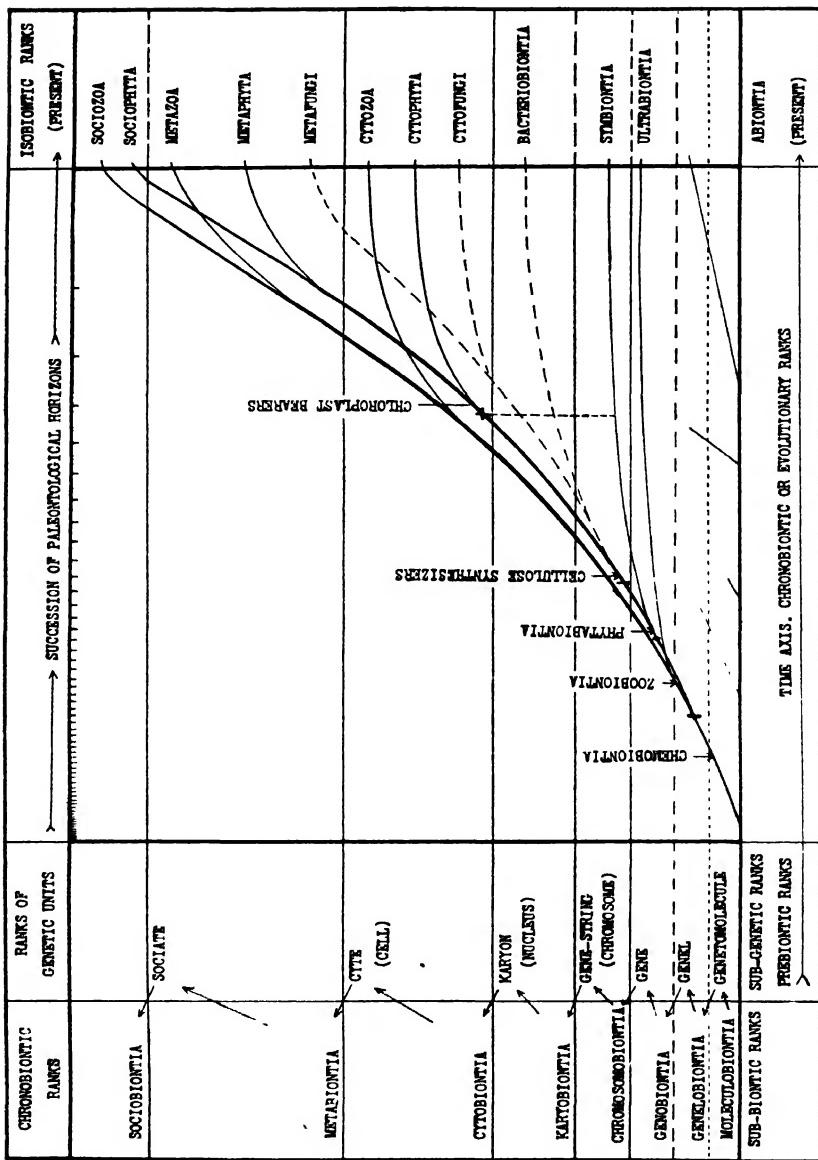
Fig. 5.—Diagram of ranks of living units.

is attained by certain Coelenterates and Bryozoans with direct relation of the Metabionts in the Sociobiont. In the social insects and in man the union is by bonds less direct but no less effective. Indeed, the enormous increase in numbers of mankind may be largely attributed to this power of combination in activity. In the plant stem, the pine forest or the Scotch-broom patch exhibits coordinated amalgamation of Metabionts into Sociobionts.

In concluding these remarks on the course of evolution, and especially its graph in Figure 5, we may add that this section represents the best estimates we can make at present, but is admittedly speculative.

THE ORIGIN OF THE FIRST MOLECULOBIONT

Troland (1914, 1916, 1917) has given the most credible account of the origin of the first molecule of living matter. It was the last step in a series of combinational interactions. Each of these forerunners was the result of the collision of two molecules with the right speed in relation to each other, and with the right surfaces presented. Each interaction was thus a chance event in the molecular chaos. We need not enter further into details here, since we have given a general account in Part I.

THE CONTROL OF EVOLUTION

The study of heredity and of cytology has given us a definite analysis of the machinery whereby new characters are produced and transmitted to the progeny. The survival of each new type is determined by selection, either artificial or natural, of that form, whether the old or the new, which is best suited to the environment in which the competing forms find themselves.

Until recently we have had to be content with selection from the new variations that occurred spontaneously in nature. We could combine the new variations with each other and with the old variations, and produce a great diversity of types.

But now a new era has been opened, largely through the work of Muller on Drosophila (1927). The finding of Muller has been extended by Goodspeed and Olsen (1927) to tobacco, and have been confirmed for Drosophila by Weinstein (1928) and Demerec (unpublished results). These experiments show that the rate of occurrence of mutations can be artificially increased by X-ray treatment to ten or fifteen times its normal value or perhaps even to 150 times that value. All the kinds of mutations, which occur normally, seem to be produced in heightened frequency through the treatment, and quite new kinds of mutative processes may be demonstrated.

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Solutions and Life *

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According to the teachings of modern physics, both colloidal and crystalloidal solutions are represented as possessing a homogeneous uniformity throughout their entire body, having the same physical and chemical properties throughout, being without differences and without structure throughout, with the exception, however, of their surfaces, where physics notes the existence of a different stratum, infinitely thin, that results from surface tension or adsorption.

Such a conception of solutions is contrary to facts found by experimental study, these ideas having resulted from imperfect observation. This notion of homogeneity and amorphousness in solutions is a serious obstacle to the progress of biology.

Living media, blood, lymph, cytoplasm, are solutions of crystalloids and colloids which do not become coagulated until after death. Life, with all its chemical and physical phenomena in these liquid media, is simply the result of differences which exist in them or which are continually produced.

An understanding of life is, therefore, dependent upon the recognition of differences that occur in solutions under all the influences which affect living organisms. Two physical methods are of special value in detecting differences in solutions, hitherto unobserved: first, very fine suspensions show currents that bring about in solutions all these effects; secondly, transparent liquids when traversed by an oblique beam of light show differences in refraction in the portions of the liquids observed, that reveal differences of structure hitherto unseen. The experimental study of solutions containing fine suspensions, such as red blood corpuscles, or carbon particles of India ink or fine precipitates formed in the liquids studied, yields at once revelations that cast a clear light on a great number of phenomena that have been hitherto the most mysterious in biology.

First of all such an examination shows us that diffusion does not take place in solutions as physics tells us, i.e., by means of a homogeneous mixing process that proceeds uniformly. Diffusion always occurs by means of currents in two opposite directions; currents of the dissolved substance in the direction of the lower osmotic pressure; currents of solvent (e.g. water) in the opposite direction, that is to say, from the lower osmotic pressure to the higher osmotic pressure. The first mentioned current may perhaps be called a descending current, the second one an ascending current.

For the sake of simplicity, let us consider in a solution, a single point of osmotic pressure that is higher or lower than that of the solution, that is, a point in which at the moment of observation the molecular concentration is higher or lower than in the rest of the liquid. Because of this, such points

become dynamic centers radiating centripetal and centrifugal forces. At the point of higher concentration, the dissolved substance yields to these forces and moves in a direction radiating centrifugally, the water or the solvent moving in a radiating centripetal direction.

The point of higher concentration is a veritable positive pole of diffusion; the point of lower concentration a negative pole of diffusion. The existence in solutions of these centers of force or poles of diffusion is admirably revealed with their centrifugally and centripetally radiating fields of force, simply by letting fall into such a solution a drop of defibrinated blood or India ink. Immediately one sees at the center or pole, the suspension in the act of orienting itself following the direction of the radiating forces. One thus obtains a representation of the field of force of diffusion, and this field is similar to that obtained in the case of a magnetic system through the orientation of iron filings on a white piece of paper.

Figure 1 is the photograph of a center of force obtained by means of a fine suspension; it is the photograph of the field of force of a dynamic osmotic center.

Figure 2 is a photograph taken by means of difference of refraction of a field of force with a dynamic center formed by means of a little piece of sugar dissolving in distilled water.

Figure 3 is a photograph (obtained as usual by refractive differences) of a field of force produced by a drop of distilled water in salt solution.

Fig. 1.—Monopolar diffusion field; a drop of blood in hypertonic serum.

The experimental study thus reveals the production and behavior in solutions of dynamic centers of force or poles of two kinds, those with the higher pressure than the medium as positive poles, and those with lower pressure than the medium as negative poles.

If in a solution we form two similar dynamic centers close to each other, two positive poles or two negative poles, we see that their lines of force are mutually repellent in a manner similar to the lines of force of two magnetic poles. If, on the contrary, we form close to each other in a solution, two poles of opposite sign, one of higher concentration as the positive pole and one with a lower concentration than that of the solution as the negative pole, we see the lines of force radiating from one pole, bending, directing and concentrating themselves toward the other pole.

Figure 4 is a photograph of fields of force between two poles of diffusion of the same sign, and Figure 5 is the photograph between two poles of diffusion of opposite sign, one positive and one negative. They show that these fields of force are similar to those between two magnetic poles of the same sign or, as the case may be, similar to two magnetic poles of opposite sign. This
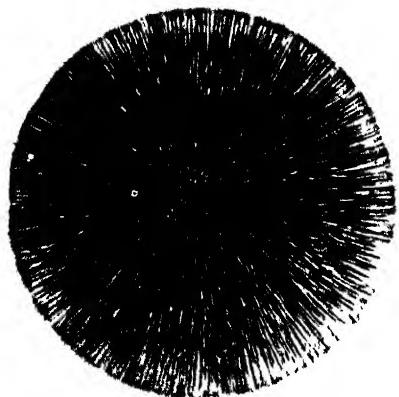
experiment reveals the same sort of dynamic relations. In this way, we have arrived at the discovery that in solutions and in living organisms there are centers of force, dynamic centers and their fields. These centers and their fields of force are similar to magnetic centers and magnetic poles with their fields of force; the same sort of dynamic relations appear between poles of same or of opposite signs. In other words, the dynamic centers or poles of diffusion in solutions follow the same laws as those which regulate magnetic poles. All the laws that have been discovered after so much labor and have been elaborated for magnetic poles and fields and which have contributed so much to the progress of magnetism and electricity, are found to be applicable to diffusion in solutions. These

FIG 2.—Diffusion field of a piece of sugar dissolving in water.

• dynamic centers of centripetal and centrifugal forces that we have come to recognize in the diffusion of liquids and in magnetism, may be found in a great number of other phenomena: An electrical charge, for example, is a center of centrifugal forces in the case of charges of the same sign, and centripetal forces in the case of charges of opposite signs. A crystal that dissolves or that is formed in a solution, is a dynamic center of centripetal and centrifugal forces. Each point of an electrode in an electrolyte is a centripetal and centrifugal dynamic center. Suns are dynamic centers of centripetal force which prevent planets from flying off into space, and are also centers of centrifugal force which prevent any planet from falling into its sun.

Finally, all living cells show a dynamic center, centripetal and centrifugal, and such activity brings about absorption and elimination. Because of the similitude of so many phenomena, the dynamics of the whole universe appear more simple.

FIG. 3.—Diffusion field of a drop of pure water in salt water.

We have considered in colloidal and crystalloidal solutions, how centers

of force are determined by differences in osmotic pressure and by differences in concentration.

The same methods of experimentation reveal to us, how every point where any action takes place in a liquid, becomes a center of force, the field of which extends into the liquid.

Figure 6 is a photograph of such a center and field of force brought about in a solution by the simple contact of a glass rod.

Figure 7 is a photograph of the center and field of force produced by the contact of a glass rod covered with paraffin.

Solutions are extremely sensitive to external effects; volatile substances, odoriferous substances, light, the least difference in concentration, color, heat,
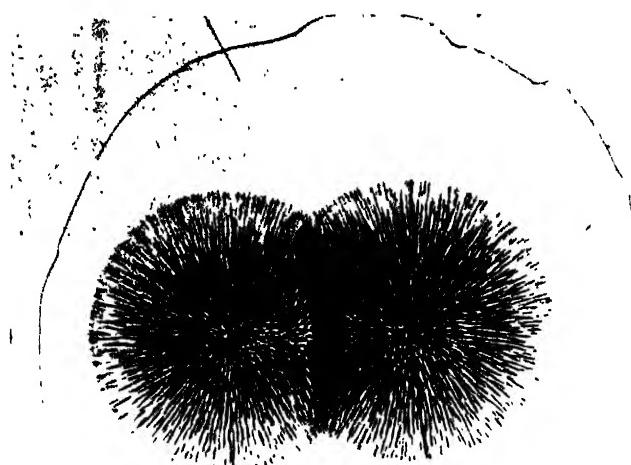
Fig. 4.—Diffusion field of two poles of the same sign. Two drops of blood in hypertonic serum.

etc., brings about a change in the topography of forces (the production of currents) and in the morphology, that is, the structure of solutions.

These experimental researches show colloidal and crystalloidal solutions to be very different from the way in which they are conceived by modern physics. Instead of being uniform, homogeneous, amorphous, insensitive; instead of being inert reservoirs of osmotic pressure, of stable potential energy, existing in the static state, experiments show us that solutions continually differ within themselves, are sensitive, react to all sorts of external effects; instead of being stable reservoirs of potential energy, experiments show us that solutions are transformers of energy in continual activity, receiving exciting stimuli from without, heat. light, etc., and responding to such stimuli by various transformations of energy, producing mechanical energy in the

form of currents, of radiating forces differing from those that have been received, of chemical energy in solutions chemically unstable, etc.

All these changes and transformations determine in solutions morphological differences, which show, through differences of refraction, that solutions are not amorphous as has been believed. Experiments, particularly the examination of their refractive differences, show continual changes and very diversified structures in solutions.

Figure 8 is a photograph of a solution of tri-sodium phosphate on the surface of which a thin layer of water has been carefully run. Figure 9 is a photograph of a solution of nitrate of soda under the same conditions. Liquids, the photograph of which in an oblique beam of light shows these

Fig. 5.—Diffusion field of two poles of opposite signs, formed by a hypertonic drop and a drop that is hypotonic to the medium.

structures, have upon a direct examination a uniformly transparent, homogeneous and amorphous appearance.

• We have stated that the discovery of centers of forces in solution with
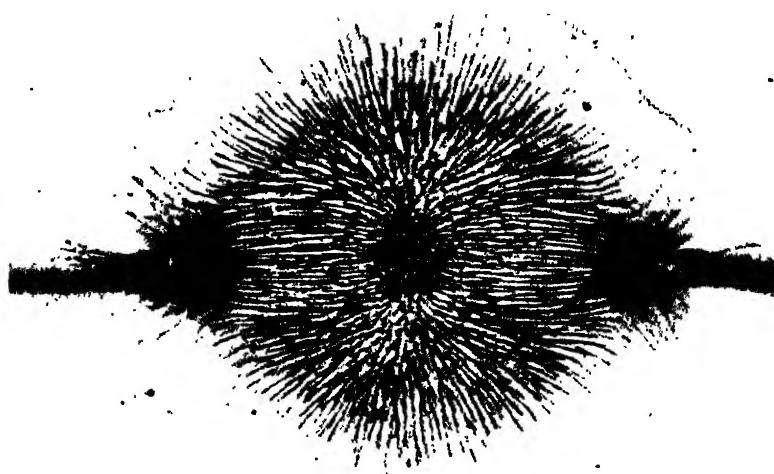
Fig. 6.—Field of contact of a glass rod with a drop in the process of diffusion.

their fields has thrown a bright light on some of the most mysterious phenomena of life. This is particularly apparent in the case of the curious forms and in the motions of karyokinesis.

The discovery of this formation by Herman Fol caused a great surprise.
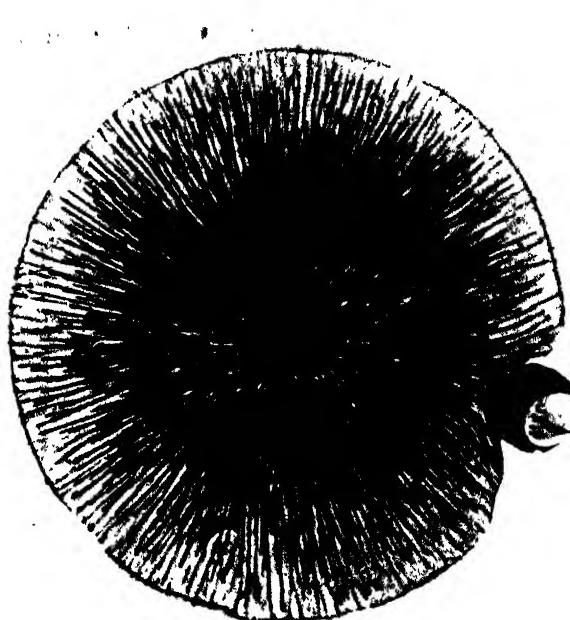
FIG. 7.--Field of contact of a paraffined rod with a drop in the process of diffusion
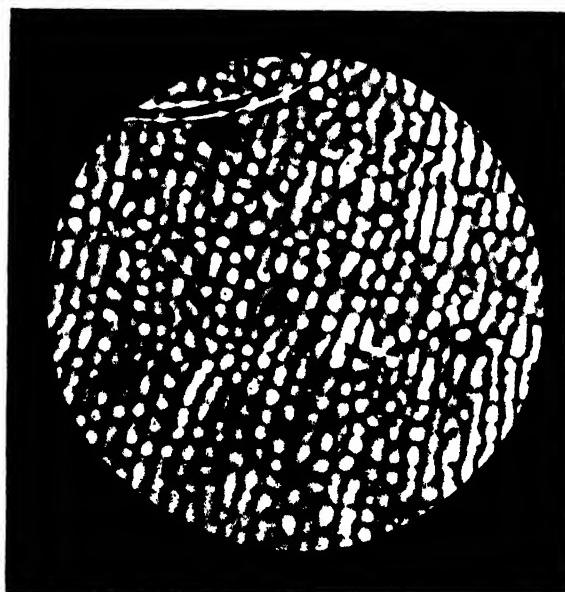
FIG. 8.--Surface separating a solution of tri-sodium phosphate and a layer of water.

Fol at once recognized the resemblance of those forms to magnetic fields; he says "The whole figure is extremely clear and recalls in a striking degree the appearance and methods by which iron filings arrange themselves between the two poles of a magnet." "I adhere," he states, "to the theory of Sachs which states that this division takes place through centers of attraction. My belief of this idea is not determined by theoretical considerations, but because I am able to see the centers of attraction." But at that time no center of force in a solution was known; it was not known how centers of force could be produced, and in order to interpret the observed facts it was necessary to have recourse to all sorts of hypothetical forces, to magnetic force and to electrical force because of the resemblance of these forms; for these were the
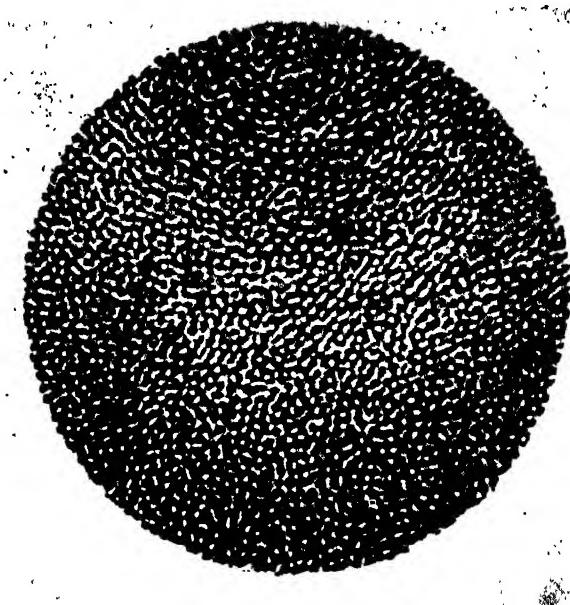

Fig. 9.—Surface of separation between a layer of water and a solution of sodium nitrate, illuminated by an oblique light.

only forces then known to give to their fields the forms that are seen during the process of cell division. Recourse was also had to mechanical forces. No one had ever seen in aqueous solutions such as compose living organisms, any form with a magnetic aspect. No one had any idea how similar figures could be produced in aqueous solution. By my experimental study on diffusion, I showed how all the representative forms of magnetic and electrical fields could be obtained with relative facility in aqueous solutions. I was able from that time artificially to reproduce the curious phenomena observed in cells during karyokinesis. The success obtained exceeded our hopes, for not only did the experiments permit our reproducing the appearance and the forms of karyokinesis, but their complexities and their peculiarities took place in consecutive and regular order. All the movements, all the transformations, all the successive different and varying forms from the appearance of centrosomes

and asters till the formation of chromatic nuclei, the forms of the spireme, of the spindle, the equatorial belt, chromosomes, up to the final formation of the two daughter cells.

Fig. 10.—Reproduction of the appearance of karyokinesis by means of the diffusion of liquids.

The artificial karyokinesis reproduced under the guidance of the conception of dynamic centers gives in solutions, a real motion picture of natural phenomena. Figure 10 is a spindle between two centrosomes surrounded by their asters artificially produced and photographed at the moment of their formation. To show the great variation of successive forms and the perfection of this reproduction of nature in artificial karyokinesis; Figure 11 is a photograph of an artificial nucleus corresponding to the period of formation of the spireme and the chromatin skein. During the formation of the artificial figure one may see all the peculiarities, all the details described by biologists in the natural phenomenon.
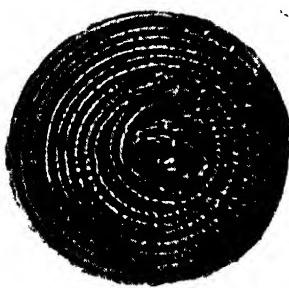
Fig 11.—Point during the diffusion of a drop of liquid, corresponding to the formation of the spireme.

Not only can we reproduce by centers of diffusion all the dynamic phenomena, kinetic and morphological, of karyokinesis, but the recognition of dynamic centers of diffusion and of their fields and behavior, permit of artificially reproducing all the anomalies of karyokinesis, that is to say for example, one can produce triasters and polyasters. The recognition of dynamic centers in solutions, of their effects one on the other, and all their inter-actions with external forces, throws a clear light on the morphology of living organisms and

on the histogenesis and structure of tissues. Every living cell is, in effect, a dynamic center of centripetal forces of absorption and centrifugal forces of elimination. Now if similar dynamic centers are made to bear one upon the other, such as often occur in nature, one obtains a resultant balance similar to a tissue of polyhedric cells even in their details like cells of living organisms. Figure 12 is the photograph of such polygonal cells entirely fluid, obtained simply by the action, one on the other, of centers of diffusion produced by dropping into salt water drops of liquid more or less concentrated and slightly colored. Figure 13 is a cellular tissue made by allowing drops of a solution of potassium ferrocyanide placed close together to diffuse in a solution of gelatin. I cite here as an appreciation of these results, the letter by which the biologist, Prof. Yves Delage, acknowledged receiving some such prepara-
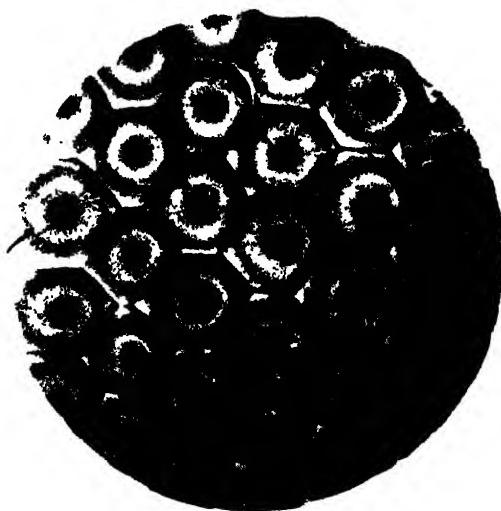

FIG. 12.— Completely liquid cells produced by the reciprocal action of dynamic diffusion centers.

tions that I sent to him: "Thank you for your beautiful preparations of cellular tissue, but I judge them to be artificial because the cells are more than perfect."

When one becomes familiar with the action of dynamic centers, it is very easy to understand how all the varieties of cells and tissues are formed, and how such forms can be experimentally realized.

The means described above, of producing dynamic centers in liquids, are by no means the only ones that intervene in solutions. In cases of fine suspension or in cases that fluids produce, by their reciprocal action, a light colloidal precipitate, one sees very, very slow diffusion progress; cohesion intervenes and by this factor, combined with regular diffusion currents, one sees produced regular division, centers of cohesion that are true dynamic centers and which produce cellular tissues similar to those of living organisms.

This production of cohesion centers by slow currents of diffusion constitute the physical mechanism of those phenomena known as glomerulisation, segmentation and agglutination.

Figures 14 and 15 are photographs of the effects of cohesion, and we see here the physical mechanism in action in the phenomena of life. It is sufficient to raise the temperature of an egg, to bring about in the egg the phenomena of glomerulisation and segmentation which unite to form the chicken: so if we follow the chain of physical phenomena, we see that the increase of temperature brings about an evaporation, and an increase of concentration at the surface of the egg. This increase of concentration at the surface regulates the slow currents of diffusion from the liquid in the center to the surface, and of dissolved substances at the surface, toward the center. This experiment shows us that diffusion currents under these conditions give rise
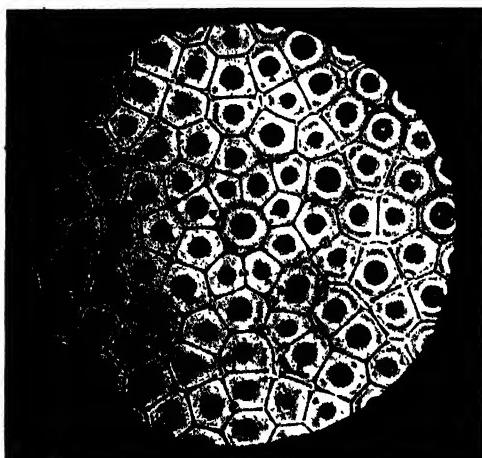

FIG. 13.—Cells produced by diffusion in gelatin solution
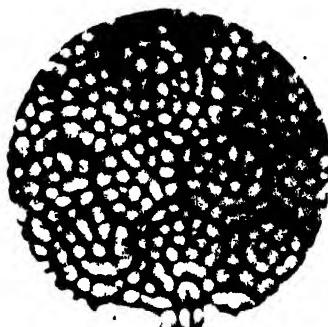
FIG. 14.—Glomerulisation or segmentation in liquid cells by very slow diffusion.

to the glomerulisation of the mass, to its segmentation and to its production of cellular tissue, which is precisely what we see produced in the egg. Biologists and physicians recognize the segmentation of liquids in living organisms. They utilize agglutination, glomerulisation, precipitation. They not only fail to study the physical mechanism of these processes, but they also abstain from making studies in the direction which, in their opinion, cannot have any connection with vital or mysterious phenomena or with incomprehensible mechanisms which occur and which they interpret by taking refuge in figures of their imagination, agglutinins, precipitins, etc., functioning somewhat like pagan gods who in ancient times produced, by their intervention, all natural phenomena.

Experiments on diffusion, on the production of dynamic centers, on their reciprocal actions, on karyokinesis, on the production of cells, on glomerulisation, on segmentation through cohesion or by precipitation, are the most beautiful in physics. I have often projected such phenomena on a screen and

these movements, these evolutions, these successive changes of form are always a source of wonder to spectators.

. Biology has not made progress comparable to that of other sciences. During centuries a continual and enormous amount of work has advanced the subject only a little. It is easy to see the causes which contributed to render these efforts futile and have retarded the progress of biology. For centuries innumerable investigators have looked for life where none existed, viz. in death. Cadavers have been dissected, microscopic sections, prepared and fixed, have been studied. It was thought that life was being studied; instead death was studied. Since all efforts have been devoted to studying life where it did not exist, it is this factor evidently that has hindered a physical conception of life corresponding to reality. Proof of this fact lies in the inability of biologists to define life. They even assert that it is impossible to describe life, that is to say, its explanation is inaccessible; therefore, why study it?

Life is the function of living cells. The living cell is fluid, the dead cell is coagulated. Life is the sum total of energy phenomena which occur in the liquids of living cells in the colloidal and crystalloidal solutions. The conception of life is therefore subordinated to an understanding of energy phenomena in liquids and in particular in colloidal and crystalloidal solutions in which this article is an elementary departure, representing the way, and the first step, in that direction which shall lead us to the comprehension of life. The cell is the elementary form of the living organism; a living organism may be formed from

Fig. 15.—Formation of a precipitate in a drop of liquid by the solution into which it diffuses.

a single cell in which one finds concentrated all the characteristic functions of life.* Cellular energy is the essential energy of life. All living cells have a common function which is found in every cell of mono-cellular organisms, viz. nutrition. Nutrition is a fundamental function that is characteristic of life. In the process of nutrition foodstuffs enter into the cell from the outside and waste products are discharged in an outward direction from the inside of the cell. A living cell is therefore a dynamic center in a liquid medium like those we have experimentally studied. But in bringing more or less concentrated drops into liquid media, we have ourselves brought about a force, an animating energy of dynamic centers. What fulfills this rôle in the living cell? Observation shows us that it is the energy surrounding and acting upon living organisms that excite the liquids of which they are formed. The living cell is the transformer of energy, the function of which from birth to death is maintained by stimuli coming from the outside. The living cells not only receive their substance from the outside but they also

* See, however, F. d'Herelle, this volume. *J. A.*

receive all the energy that animates them, not only the energy of food which they transform but also the energy of stimuli which brings about the changes produced in liquids that are formed by living organisms, and also the movement and chemical reaction of which they are the source. However, the direction to follow in order to arrive at an understanding of life is clear, and can be investigated by experiments. It is necessary to study the behavior of liquids which are the same as on those which form living organisms (that is, colloidal and crystalloidal solutions), under the stimulus of all kinds of external energy.

In living organisms the centers of chemical action, of synthesis, or of decomposition, are dynamic centers. The physical and mechanical rôle hitherto not understood in these areas is foremost in the mechanism of life; it is the direct transformation of chemical potential energy to mechanical energy that animates living organisms. The phenomena of chemical actions in liquids have hitherto been studied as a whole as if they occur everywhere. Nevertheless, in living organisms we see chemical actions distributed in foci in every cell. We must now consider these chemical centers of force in solution and seek how we can bring about these centers; how they evolve and what are their effects. Figure 15 shows two liquids reacting one on the other to form a precipitate, and demonstrates well the separation into zones or foci in this case.

The chemical zones or foci in solutions are those dynamic centers having all the properties, all the actions, all the effects of centripetal and centrifugal centers of force. In every synthetic center where a number of molecules combine to form one, the molecular concentration and the osmotic pressure vary in proportion to the number of molecules, from each center of decomposition or where one molecule decomposes into many, the molecular concentration and the osmotic pressure rise in proportion to the number of molecules. A chemical focus is the center of centripetal and centrifugal forces, the form and field of which are similar to those in living cells.

It is very clear that this transformation into foci of syntheses or of decompositions of chemical energy, is, in solutions which constitute living organisms, the transforming energy that excites them, which produces the force, the movement, the animation of living substances following the laws of centripetal and centrifugal dynamic centers. Living organisms absorb with their food potential energy which is taken up and transformed within them; but an occurrence of the highest importance, scarcely recognized by physics, takes place at this point. This occurrence is completely ignored or rather completely unknown in biology, where, nevertheless, it plays an essential rôle, and it is that no potential energy is taken up or transformed until a small quantity of outside kinetic energy, exciting energy, or unlocking force begins to act on it. Without this intervention, without this stimulating energy, potential energy continues indefinitely without becoming available, without being transformed. In the case of man the exciting energy is that which affects the senses. It is the same for all living organisms. It is so in the case of the living cell. This process of unlocking potential energy by means of exciting energy is, however, essential to life.

To recapitulate, life is the function of dynamic centers of force in colloidal and crystalloidal solutions, functioning by means of external stimuli which bring about syntheses and decompositions.

The study of exciting energies on living cells; the study of reactions, of

exciting energies in colloidal and crystalloidal solutions, in particular those which produce chemical reactions, is that which will contribute most largely to the progress of biology.

In living organisms as well as in some inanimate bodies, certain substances bring about chemical reactions without themselves participating in any way; they are the same after the action as they were before. These reactions are called "catalytic reactions," "reactions of presence," and such agents are particularly the colloidal metals and, in living organisms, enzymes or soluble ferments. The mechanism of these reactions so widely distributed in nature, has remained up to the present time very mysterious and no satisfactory interpretation of them has ever been given. The rôle of exciting energies permits us to interpret, to comprehend the rôle that catalysers and soluble ferments play by their presence. All bodies receive under various forms the energy of their surroundings, and give to their media all or part of this energy. Those which give up only a part of the energy received, use the conserved portion to modify themselves through chemical or physical metamorphoses. Those which give up to their media all the energy received, undergo no change themselves, but they generally transform the energy to a form or to forms different from those that they have received. On the other hand, if the energy received is unsuited to change the media, the energy given up by the transforming substance may, through its new form, bring about chemical reactions in the surrounding media, and this reaction energy corresponds exactly to the "reactions of presence" of catalysers and of soluble ferments. Catalysers are transformers of energy, transforming surrounding energy to energy fit for accomplishing reactions between substances in the media where they are found; they receive energy in the improper form as exciting energy, and they change it into a new form suitable to effect the discharge of potential energy in the media where they occur.

Catalysers and soluble ferments do not participate chemically in reactions, but they participate physically by giving up the surrounding energy in the necessary form to act as exciting energy.

Structures having parallel curves, either circular or rectilinear, are very common among living organisms. Cross-sections of vegetables, of trees, starch grains, etc., show concentric lines. Almost all shells show concentric and parallel lines. Mother-of-pearl is formed from calcareous layers having parallel stratifications; cartilaginous cells, tendons, muscles and many other tissues show parallel lines in their structure.

We have been led to believe that in organic liquids, tissues are formed by the action of centripetal and centrifugal dynamic centers, the fields of which are similar to magnetic or electric fields. In these last mentioned fields, the sum total of spherical equipotential surfaces, curves or planes, following the direction of force, reproduce those stratified structures that occur frequently in living organisms. By means of the play of diffusion forces, we have obtained and have been able to photograph the lines of diffusion force in a great many diversified forms under a great many diversified conditions. It is equally easy, by means of diffusion forces alone, to obtain the stratified structures that are formed by equipotential lines. In 1901 we presented at the Congress of the A.F.A.S. at Ajaccio, stratified structures formed by means of periodic precipitations that were obtained by causing a solution of potassium ferrocyanide to diffuse in gelatin that contained traces of ferric sulfate. In 1907 at the meeting of the A.F.A.S. at Rheims we presented wonderful strati-

fied structures obtained by means of materials that are found in all living organisms, namely, by causing solutions of sodium carbonate or tri-sodium phosphate to diffuse in gelatin containing traces of a calcium salt. The distance between the surfaces obtained varied from about a millimeter, in the case of the carbonate of soda and a low calcium concentration, to one-thousandth of a millimeter with concentrated tri-sodium phosphate. With mixtures of sodium carbonate and tri-sodium phosphate all gradations of differences between the two extremes were obtained. In this way, a synthesis was made and an organic tissue was artificially produced, which up to the present time has not been formed except by living organisms, because the stratified precipitations of tri-calcium phosphate and of carbonate in the gelatin, possessed the structure and physical properties of mother-of-pearl. They had a brilliance to the eye that could rival genuine pearls and by forming this precipitation on panes of glass, I prepared beautiful tracery by which I was able
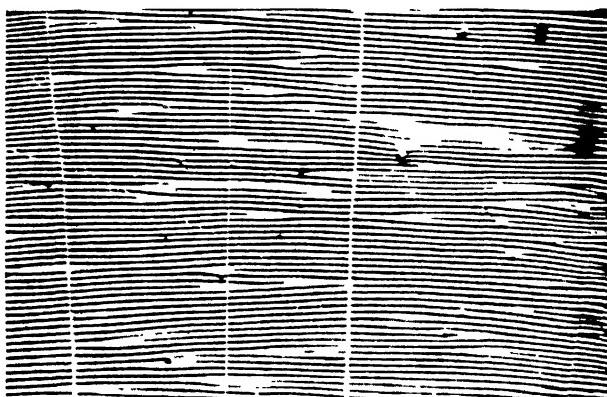

FIG. 16 —800 fold magnification of the structure of artificial mother of-pearl
formed by deposit on glass

at the Rheims Congress in 1907 to project on the screen a very marked luminous spectrum. Figure 16 is a photograph of this tracery. Periodic precipitation has been noted by a certain number of scientists among whom is R. E. Liesegang, and German authors give it his name.[*]

One of the most pernicious errors that is taught by classical treatises on the mechanics of solutions, is the statement that colloids do not influence the phenomenon of diffusion, which, it is claimed, takes place in such solutions as if the colloids were not present. It is extremely easy to show by experiments that the presence of colloids has the greatest effect upon the rapidity of diffusion and that the more concentrated the colloid solution is, the more slowly the same saline solution diffuses. This fact is necessarily of great importance in living phenomena. In reality, currents of diffusion follow the same laws as electric currents; their speed or intensity is proportional to the osmotic pressure (difference of potential) and it varies inversely with the resistance. The difference is that the coefficient of resistance depends not only upon the medium in which the diffusion takes place, but also upon the

[*] See paper by R. E. Liesegang, Vol. I of this series. Also paper of H. Schade, this volume. *J. A.*

diffusing substances. Among the many experiments showing the effect of colloids on the rapidity of diffusion we have selected one that is particularly striking and suggestive.

We have remarked how widely distributed in nature are examples of centripetal and centrifugal dynamic centers, shown by lines of force radiating in all directions from a center and by equi-potential surfaces perpendicular to the lines of force which are spherical and parallel to the dynamic center. Newton conceived light to be a center of emission for particles flying off radially. The graphical representation of a field of luminous force is thus similar to that of our dynamic centers. Huyghens conceived light to be a center of emission of spherical waves, whose graphical representation, formed of spherical, concentric, and parallel surfaces, is similar to the representation of a dynamic center with equi-potential surfaces. In interpreting the phe-

nomena of interference and diffraction, modern physics admits the undulations of Huyghens and for the interpretation of electrical phenomena, physics admits the emission of electrons by incandescent bodies. Modern physics presents the theory of emission and the undulatory theory as being distinct one from the other, although it admits that the two may occur simultaneously. Now in the case of solutions forming periodical precipitates, we show united in the same physical phenomenon the process of emission and that of undulation. Molecules that diffuse like Newton's particles or like electrons, recede from the center in radiating and rectilinear directions. The molecules form by these periodical precipitates, spherical, concentric and parallel surfaces which, like the waves of Huyghens, refract, interfere and diffract.

Fig. 17.—Spherical diffusion waves refracted and transformed into plane waves by more concentrated gelatin solution The trajectories of the diffusing molecules representing the rays, are transformed from being divergent to a parallel condition.

Figure 17 is a photograph of an experiment which shows how a drop diffusing in a solution produces physical effects analogous to an incandescent point that radiates light. Here the molecules recede from the center of the drop in radiating and rectilinear directions, like electrons from a luminous body. It is possible to see that the molecules of the drop in radiating have produced spherical waves that are concentric and parallel, and are alternately transparent and opaque. On their course we have placed a convex dioptric separating the gelatinous solutions of emission from a more concentrated gelatin solution and it is possible to see that this dioptric changes the spherical front of the waves to a plane front, and consequently transforms the bundle of rectilinear rays that are divergent and concentric (being so formed by the direction of the molecules) to a bundle of rectilinear rays that are parallel. We have been able in this way to produce by means of molecules acting in a solution all the optical phenomena that are produced by electrons acting in transparent media. How far we are from the diffusion that modern physics

Fig. 18.—Shell-like growths produced by osmosis.

teaches, and how these facts show what one may justly hope for from experimental investigation of solutions! As with light, the refraction in passing from one transparent medium into another, depends on the speeds of propagation in the different media, on the electrons, or on the waves; that is to say, the light. So also Figure 17 shows how molecules that diffuse do not have the same speed in colloidal solutions of different degrees of concentration, contrary to the teaching of contemporary physics. Variations of concentration of colloidal solutions affect the movement of liquids that takes place within them; the variation of colloidal concentrations consequently influence all living phenomena.

The physical study of the reciprocal actions of solutions, particularly of those producing precipitates, is indefinite in extent and we cannot here detail all of our findings.

FIG. 19.—Osmotic growth.

Osmotic phenomena discovered by Abbé Nollet with the aid of pig bladders, whose work was repeated by Traube with membranes formed through chemical precipitations, are well known. The phenomenon of osmosis, under certain experimental conditions, by means of a mechanism analogous to that acting in the development of living organisms, gives rise, through gentle and progressive development, to an infinite variety of formations. These formations by their growth, their shapes, and the phenomena that they show, coincide remarkably with living forms, and rightfully claim a thorough-going and minute investigation. Osmotic formations have a duration of growth, development, and evolution, that constitute their life cycle.

Influences that affect these phenomena in their development and can definitely arrest them, produce disease or death. For example, if one withdraws an osmotic formation from its developing medium, the formation is arrested on account of lack of materials to be absorbed, that is, by inanition. Now, if this formation is later replaced in its original developing medium the growth

recommences, but the osmotic product carries in its formation the mark of the period of inanition. If the inanition is somewhat prolonged, development does not resume when the formation is again replaced in its nutritive medium;
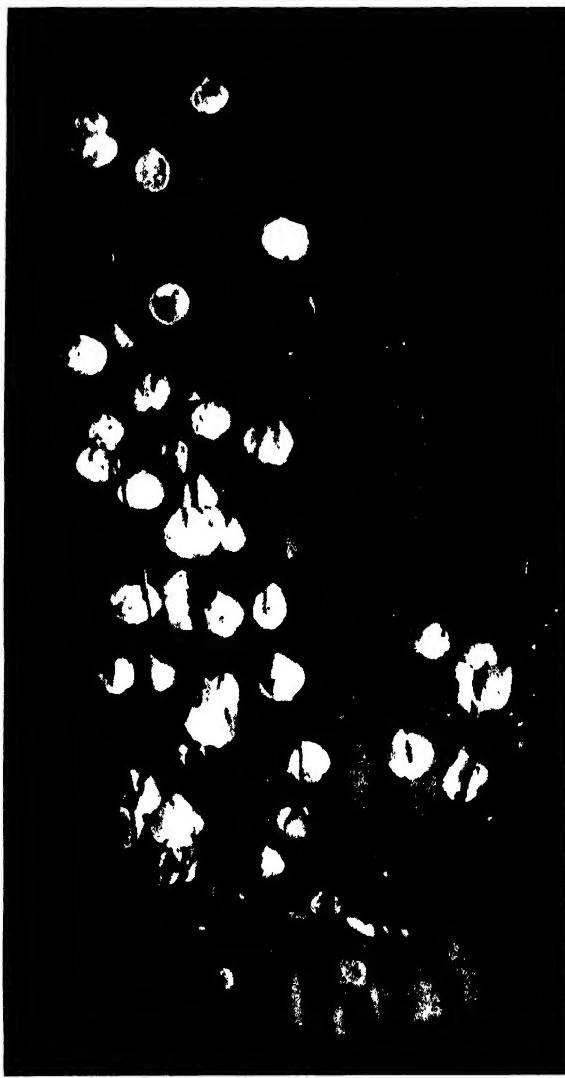

Fig 20. - Osmotic growth.

the growth is dead from inanition, dead from hunger. Most of my experiments on osmotic growths and forms, have been made with substances that form the skeletons of living organisms. The same experiments conducted on various substances which enter into the composition of living organisms certainly would give rise to the most curious results.

An elementary experiment consists in placing a bit of·pasty calcium chloride in a saturated solution of sodium carbonate. A beautiful osmotic cell is formed that swells and absorbs substance in its nutritive medium which·surrounds it. A remarkable fact is that this osmotic envelope, instead of being formed of hard, opaque calcium carbonate, is composed of a supple and transparent membrane. Such experiments can be infinitely varied. One can make them using tri-sodium phosphate and adding it to different solutions; e.g. adding as the nucleus soluble calcium salts, iron salts, manganese, etc., obtaining with each variation a different result. Figures 18, 19 and 20 are these examples of osmotic growths under different conditions.*

We have studied in colloidal and crystalloidal solutions the effects of forces which develop within them, and the exciting forces that affect them from without, and we have seen that these forces radiate from a center in every direction in the same proportion. Nothing is clearer than this fact when such a center is formed by a crystal and is made visible as the crystal dissolves or forms. Here the forces seem to be ordered, i.e., directed in certain directions, and nothing is more curious than to see a projection of this performance on a screen, as for example, during the formation of a crystal of sodium chloride, or better still of ammonium chloride.† See Fig. 21.

Figure 22 is a photograph of a crystal of sodium chloride taken during its formation in a colloidal medium and shows the regularity and the direction of forces during its formation During the crystallizing process, as during the diffusion process, the colloids present a resistance, a conflicting force that disturbs and modifies the process by giving a resultant force different from the results of direct force. The forms resulting from the resistance that the colloids oppose to crystallization, recall in a remarkable manner the shapes of plants.

Fig. 21.—Crystal of sodium chloride formed in a colloidal medium.

Figure 23 is a photograph of such a shape. Since plants are formed in colloidal and crystalloidal solutions, the forces and the phenomena observed must necessarily intervene in the formation of plant structures.

We mention here a curious phenomenon that occurred during the study of crystallizations :—

While studying crystallization that was taking place in a very thin layer of solution, by means of a divergent pencil of light reflected from a mirror at a 45° angle, we saw a formation appear that resembled the lines of flow in a magnet. Surprised at the bi-polarity of this figure, we studied the formation of crystals of different systems, and we always found at the beginning of crystallization, that there was an appearance of a bi-polar field. We then substituted for the solution a thin layer of distilled water, and for the crystal,

* The reader will find these numerous results and developments in my works "La Biologie synthetique et l'Energetique de la Vie," "Physico-chemical Theory of Life and Spontaneous Generation," Poniot edit. 21 rue Cassette, Paris.
† Important, but generally overlooked work of Ramey, Ord and others, is given in "The Effect of Colloids on Crystalline Form and Cohesion" by Dr. William M. Ord (London, 1879). *J. A.*

FIG. 22.—Crystallization of sulphate of magnesia in a colloidal medium.

FIG. 23.—Field of interference formed about a crystal at the beginning of the crystallizing process.

a fine droplet of mercury (or a very fine grain of lead) and we obtained the same bi-polar field. It must be admitted that this is a matter of optics, an interference phenomenon analogous to Newton's rings. The bi-polarity may be the result of the polarization by reflection from the mirror. The interference occurring in the plane of incidence and not occurring in the perpendicular plane, determines the polar line that is always perpendicular to the plane of incidence. There is reason to ask if these optical images produced by thin layers are not those that have been used to prove the existence of liquid crystals. Figure 23 is the photograph of this phenomenon.*

* Regarding the structure of liquid crystals, see paper by G Friedel, Vol. I of this series. *J. A.*

Plasmogeny

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Plasmogeny is the name proposed by the author for a new science, which includes a study of the life-like behavior of artefacts, as well as all researches and theories which serve to throw light on the origin of protoplasm (from *protos*, first; and *plasma*, form).

From a study of artefacts and their behavior and "life," reasonable inference may be drawn as to the factors at work in living cells, tissues, and organs.

Plasmogeny makes use of the data and conclusions of many other sciences. From chemistry it appropriates knowledge about proteins, nucleoproteins, carbohydrates, lipoids, and all organic and inorganic substances found in living matter. From physics and physical chemistry, it takes what is known of colloids and colloidal behavior, including structure, surface tension, diffusion, osmosis and osmotic pressure, adsorption, electro-capillarity, electric charge, etc. From biology and its subordinate sciences, such as cytology and physiology, it draws data concerning the behavior and function of living matter (protoplasm, nucleus, cell, tissue, etc.). Hence the following divisions:[1]

Morphology { Cytogeny, or imitation of cells.
Histogeny, or imitation of tissues.
Organogeny, or imitation of organs.

Physiogeny, or imitation of functions

Chemiogeny, or imitation of organic molecules concerned in life processes, e.g. polypeptids, proteins, lipins, cellulose, chlorophyll, and such substances as vitamins, hormones, bios, etc.

The illuminating work of Pasteur showed that ordinarily there is no such thing as "spontaneous generation," the production of organisms without parents or antecedents from inorganic or decomposing organic matter, in culture media.*

This led to the aphorism: *omne vivum ex ovo*, which has since dominated biological science, although Tyndall, for example, said that he saw in matter the potentiality and possibility of all life. In fact, on reflection, it is evident that protoplasm and life must have been the results of natural energies and forces in the primitive environment, which might well have been different

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[1] For more general application of the philosophical principles of Plasmogeny, see publications by A. L. Herrera, e.g. "La Vie Universelle." *Bull. Assoc. Int. Biocosmique.* Chatenay-Malabry. Paris. May, 1926. No. 1. p. 1-16—"Una nueva ciencia. La Plasmogenia." Maucci, ed. Barcelona. 1926, pp. 1-446, illustrated.

* We, to-day, can hardly appreciate the effort that Pasteur had to put forth to establish this view; but in those days "scientific" formulas were extant, which told, e.g. "How to produce three mice" by the use of a mess calculated to attract denizens of the laboratory. *J. A.*

from present conditions. We cannot, therefore, accept this aphorism, but look forward to the possibility of discovering the conditions under which some form of primitive life will arise.* Though the goal may still be in the far off future, some steps have been made. Many of the substances of living cells have been produced by photosynthesis.

Résumé of Some Results Obtained

Mr. Maynard Shipley says [2] "The recent researches of M. Daniel Berthelot show, among other effects, that the ultraviolet rays cause decomposition phenomena in most organic compounds. To the decomposition phenomena of *electrolysis* we must now add *photolysis*, which is found to have an even wider range than electrolysis, not well limited, as it is, to certain classes of bodies in solution or in a molten state. One very remarkable effect produced by photolysis is that substances exposed to the action of the ultraviolet rays will give off gases in a manner resembling what occurs in fermentation." Moore and Webster, Baly, Heilbronn and Hudson and many others studied the syntheses of formaldehyde and of nitrogenous products from inorganic substances under the influence of ultraviolet light. Spoehr, Porter and Rampsperger [3] and the author have not attained success in these experiments. I query if there is not required a condenser, a structure probably formed by inorganic colloids; this is also the idea of Ciamician.[4] Silica and the silicates produce, as inorganic colloids, an infinite series of imitations of organic phenomena, and they exist everywhere in nature and in organic matter and cells (*see* Herrera [6] and the bibliography). Silica absorbs minute quantities of radium, which acts on cells and life. Organic photosynthetic substances, e.g. formaldehyde, also form imitations of cells and mitotic figures [7]

Plasmogeny, originated by Nollet, who discovered osmosis in 1748, was a child about 1885, with the work of Butschli and Quincke To-day it is an adult in possession of its full strength and faculties. Who knows when it will reach its objective, which is the synthesis of living matter? *

* The fact that no one has yet demonstrated the spontaneous or the laboratory production of a living thing, is no argument against the view that spontaneous generation has occurred, and may indeed, be even a common occurrence The trouble has always been that instead of searching for the origin of life in its ultramicroscopic rootlets, attention has been concentrated upon living forms tremendously advanced in the scale of evolution Life did not begin by the sudden appearance of an elephant, a mouse, a flea, or any of the other forms of life which entered Noah's Ark Since Loewenhoek's time the microscope has revealed hosts of living things (bionts), both plants and animals, unknown to the writers of the Bible cal record; but even these are quite complicated, as any book on Protozoology will show The law of probability is strongly against chance grouping of the relatively enormous numbers of molecules needed to make up an amoeba or a bacterium, but much more in favor of the chance formation of a living molecule or molecular group, as described in the first paper in this volume

Obviously, the artefacts shown by Leduc and Herrera, illustrate not living things but rather many of the physico-chemical concomitants of life They enable us to form an idea as to the development of some of the curious and even weird forms we find in plants and in animals, many of which would be scouted as figments of the imagination, were it not for the fact that *they exist* The sources of life lie in the submicroscopic field, below the level of these artefacts, and are demonstrated indirectly *e g* by genetics, just as the chemist indirectly demonstrates atoms and molecules by their experimental effects *J. A*

[2] "Man's Debt to the Sun" Little Blue Book No 808 Edited by E Haldeman-Julius, Girard, Kansas, U. S. A, pp 1-64 E Slosson "Creative Chemistry," New York, 1920

[3] "The Action of Ultraviolet Light upon Carbon Dioxide and Water," *J Am Chem Soc*, 1925, p. 79.

[4] "Gaceta Medica Catalana," 1921, p. 24. *Revue Scientifique* Paris, 1921, n 6, p. 140, and n. 20, p. 603.

[6] Researches of A. L Herrera, Mexico, 1889 to 1926, 36 volumes published, 5400 experiments. "Una nueva ciencia, La Plasmogenia" Maucci, ed Barcelona, 1926, pp 1-446, about 400 engravings and plates.

[6] Herrera, *R Accad Lincei Roma* June 15, 1924, pp 510-512 Figs.

* At the 1926 Meeting of the British Association for the Advancement of Science, Miss E. S Semmens reported experiments showing hydrolysis by light polarized by colloidal particles. She and

ORGANIC COLLOIDS

Vibert varnish dropped in water. Imitation of cells and infusoria in movement by changes of surface tension and vapors of amyl alcohol.

Tannin in gelatin and water, at 70° C. Imitation of cells and microscopic beings. The calcium chloride of the gelatin is precipitated by the silica of the tannin. Firm structures. *See Bulletin de la Société Zoologique de France,* Jan., 1899, p. 20; 1902, p. 144, fig.

Oleic, capric and caprylic acids and alkalis and alkaline carbonates, in water. A great variety of myelinoid forms, structures, movements, ciliary motion, striking resemblance to Protozoa and Protophyta. Cells, nucleus,
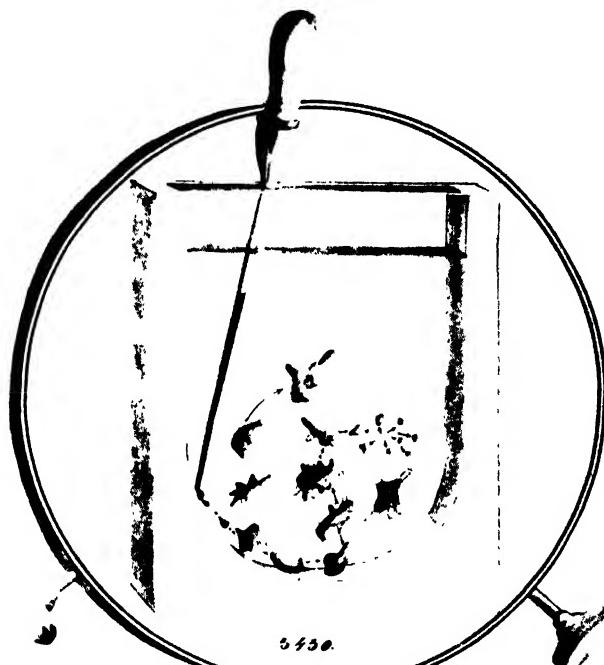
FIG. 1.- Artificial "amoebas" (magnified)

spiremes, membranes, direct division, films, palpitations, tubes, neurones, contractile threads, spirals, trees, amoeba in movement, plasmodia of Myxomycetes, ovules and embryos, etc. All the figures soluble after several days.

Colors and cross in polarized light. They are "liquid crystals." *See:* Lehmann-Herrera. *Mémoires de la Société Scientifique Antonio Alzate,* Mexico. Vol. XII, p. 242, figs.

Oleic acid in the surface of lime water. Macroscopic amoeboid movement. Palpitations, like a heart. Osmotic currents. *Bull. Soc. Zoologique France.* Jan. 24, 1899, p. 21.

others point out that this peculiar action of polarized light may be the germ of truth underlying many popular superstitions, e g , that food exposed to moonlight readily decomposes. The belief in potency of the moon also crops out in "m n-struck," "lunatic" and in certain Chinese ideographs. Miss Semmens' work has been confirmed d extended by Prof. H. I. Macht of Johns Hopkins University, reported at Richmond, Va , meeting f American Chemical Society, 1927; see *Science* for April 15, 1927 (No. 1685). *J. A.*
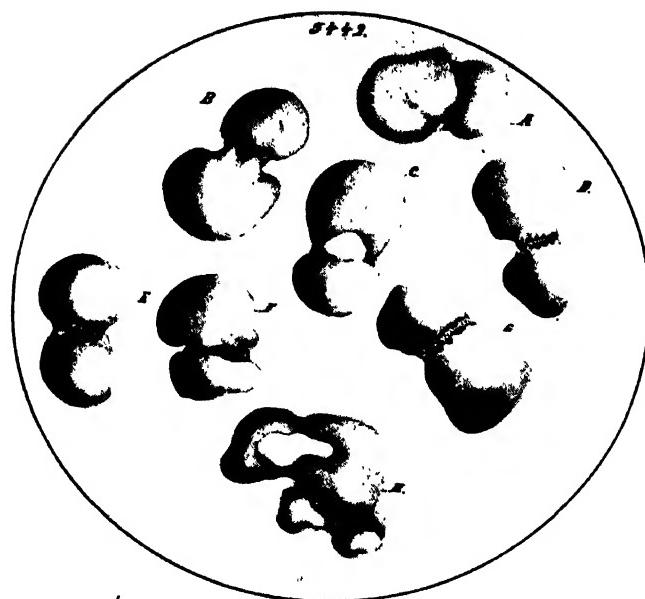
Fig. 2.—Colpoids, illustrating conflict and parasitism (slightly magnified).
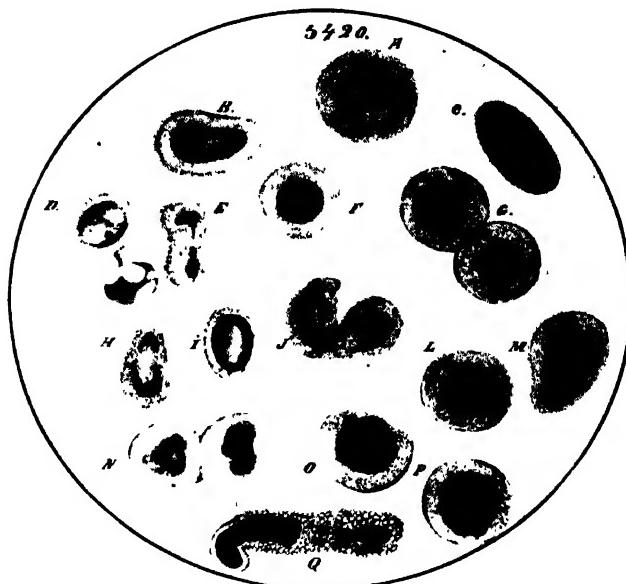
Fig. 3.—Colpoids, showing nucleus and division 24 hours after production (slightly magnified).

Oleic acid dissolved in carbon disulfide or tetrachloride, in ammoniacal water. Macroscopic amoeboid movement, vacuoles, conjugation and direct division. The same with oleic acid dissolved in chloroform. *La Médicine Scientifique de Quesneville.* Paris. December, 1911, No. 132, p. 179-182. Oil and sulfur chloride and hydrochloric acid, in alkaline water. Amoeboid movements.

Olive oil or rosin dissolved in gasoline. French olive oil (F. Bétus & Fils) 50, gasoline 100, or rosin 6, gasoline 240, and drops of a solution of caustic soda (14%) stained with rhodamine (1 gr.).

Remarkable imitations of amoeba and infusoria.—"Colpoids" (Fig. 1), artificial imperfect beings attacking and sucking themselves (Fig. 2). These osmotic cells show internal currents and a great variety of macroscopic and microscopic structures and movements, chiefly determined by changes of surface tension and the propulsive action of osmotic currents. Many functions: conjugation, palpitation, deformation, contractile vacuoles, pseudopodia, direct division and multiplication, growth, increase of surface and volume. On adding gum arabic the movements persist one hour. Adding lactose: *autophagy*, the arms of some bifurcated colpoids suck each other. On adding oleic acid, the membrane is thinnest and the pseudopodia are largest. *Phagocytosis* of carbon impregnated with acetic acid. These experiments are far-reaching and throw some light on muscular contraction and indicate some of the conditions of primary osmotic cells and membranes. Colpoids are the best imitations of living infusoria, and present the most remarkable results of Plasmogeny up to 1926. Upon desiccation or rest, colpoids show structures and nucleus in division. The same solution of soda dropped from a height of 20 centimeters on gasoline and olive oil, presents a marvelous imitation of Brownian movement, each granule being activated by osmotic currents and not by the kinetic energy of the liquid. This is a proof of the author's biological theory of Brownian movement (*La Semana Médica de Buenos Aires.* Feb. 6, 1913, p. 305-308. May 28, 1914, p. 1227-1238, figs.)

Theory of colpoids. In the photomicrographs we see the osmotic currents and the currents between two or more colpoids. Doubtless the cause of the sucking activity, resembling a vital function, is the continuous change of density of soda solution contained within each colpoid. This change is produced by the saponification by the alkali of the oil dissolved in gasoline. The density of the solution drops continuously. Then this density is not the same in two colpoids or in the different parts of a colpoid; and active currents of diffusion, by difference of density, are soon established between the artefacts and its membranes till they attain the same osmotic pressure and the same density.

This theory is also, I believe, far-reaching in biology. The pseudoplants of Traube and Leduc are also the result of two 'different densities of the exterior and the interior liquid, as probably are a great many biological phenomena. *See*, for colpoids, *Compt. rend.*, Feb. 15, 1926. *La Côte d'Azur Médical.* Toulon, France, June, 1926, No. 118-119, fig. *Bull. Muséum d'Histoire Naturelle*, Paris, 1926, No. 4, pp. 218-220, fig. *Bull. l'Assoc. Internat. Biocosmique, La Vie Universelle*, Vol. 1, pp. 13-16, 17-20, fig.[7]

Collodion diffused in a paper soaked with linseed oil. Cells and nucleus

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[7] The instability of colpoid protoplasm is like that of natural protoplasm. The death of colpoids is a result of instability.

with chromosomes. The collodion should be stained with methylene blue. Macroscopic.

Hexagonal tissue of collodion. Formula: alcohol (90%) 250 cc.; ether 240 cc.; glycerin 8.40 cc.; ultramarine blue 6 grams; ivory black 1 gram; aluminium in fine powder 5.520 grams; gold bronze in fine powder 6 gr. Cellular whirls of Dauzère, Cartaud and Bénard. Hexagonal tissue, nucleus, embryos, fine structure of protoplasm, etc. They are due to the evaporation and formation of concentration nuclei, each nucleus becoming hexagonal by pressure. If evaporation is prevented, there are no structures. For imitation of mitosis, this liquid is poured on a glass plate, a drop of water is put in the center, and strong aspiration is applied by an air pump and two tubes.
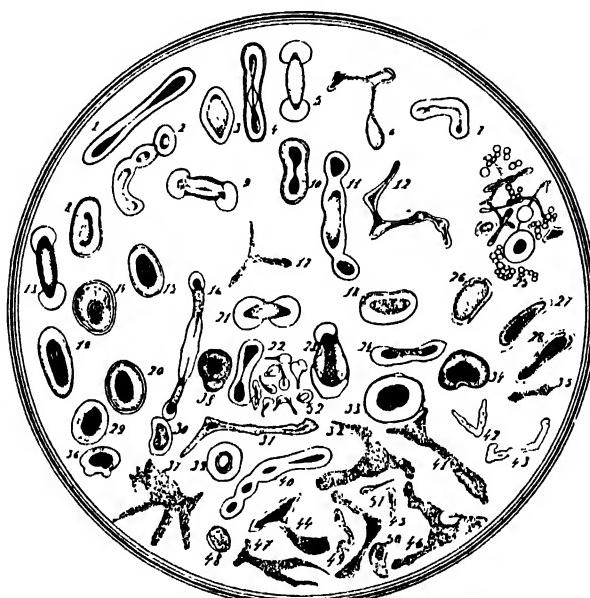
Fig. 4 — Some imitations of cells made with calcium fluorosilicate; fixed, stained, and mounted in Canada balsam (From *R. Accad. Lincei*)

Splendid asters are produced, mitotic figures, with a spindle and chromosomes. (See "Una nueva ciencia, etc.," p. 292-293, figs.)

Formol evaporated in a desiccator. Cellular and mitotic figures, imperfect crystallisation. This experiment is evidence favoring the theory of the photosynthetic origin of life proposed by Herrera in 1905, in *Revista Chilena de Historia Natural,* Aug., 1904, pp. 235-237. See *Rend. accad. sci. Lincei,* April, 1925, p. 364, and Jan., 1925, p. 5. Baudisch. *Semana Médica,* Buenos Aires, Dec. 21, 1922, p. 1319.

Gelatin and glycerin. Injection of air or gas: polyhedrical cells, tissue, imitation of insects' compound eyes, multiple images. See "Botánicä" by Herrera. Herrero Hermanos, México, editor. 1924, p. 404. Lima Scientific International Congress, 1924. Herrera, "Una nueva ciencia. La Plasmogenia," p. 263.

Albumin and alkalis. Imitation of fibers. *Bull. Soc. Zoologique France,* Jan. 24, 1902, p. 23. *Mémoires de la Société scientifique Antonio Alzate.* Mexico. Vol. XI, pp. 29-31. *Rev. Sci.,* Jan. 10, 1902.

Albumin and phosphoric acid. Movements and structures due to impurities (soluble silicates). *Bull. Soc. Zoologique France,* April 8, 1902, p. 159-164, figs. Structures of gelatin and metaphosphoric acid. *Ibid.,* June 24, 1902, p. 177-201. *Mém. Soc. Alzate,* 1902, p. 133-136, figs.

Egg albumin and acetic and metaphosphoric acids. Silicic structures. *Bull. Soc. Zoologique France,* July 22, 1902, p. 201-202, figs.

Rosin and gasoline (20: 2.50) and drops of soda solution (14%). Microscopical amoeba, retractile pseudopodia. Soluble in gasoline. This is the best imitation of amoeba produced in a laboratory.

INORGANIC COLLOIDS

Sodium silicates, colloidal silica, organic compounds of silica and fluoro-silicates mixed with salts, alcohol, sulfuric ether, acids, formol, oil, etc Silica is found everywhere. Cells, animal and vegetable tissues in thin sections, slowly incinerated, show silica. Also in organic matter (tannin, proteins, nucleins, crystallised chemically pure sugar, etc.). Probably silica is a constituent of organisms. *See* Herrera, "Sur la presence de la silice dans les coupes histologiques incinérés." *Compt. rend.,* Feb. 16, 1925, p. 538. "Biologia y Plasmogenia," México, 1924, p. 245-286, with the full bibliography of the matter. Silica is the universal colloid and shows morphological and diastasic properties. *See* Zsigmondy, "Coloidequimica." Calpe,

FIG. 5 – "Spores" made with calcium fluorosilicate.

Madrid, 1925, p. 267-297. Perhaps silica is the substratum of life. It adsorbs radio-active substances (Ebler and Fellner, *Z. anorg. Chem.,* **73,** 1-30 (1912)). The best imitations of mitosis are produced with silica and substances rich in silica, *e.g.,* collodion and oils. An extensive series of models produced by silica and fluorosilicates may be found in the author's publications. *Compt. rend.,* May 19, 1919, p. 388, June 28, 1920, p. 00. Importanza biologica dei fluosilicati. *Rend. accad. sci. Lincei,* Jan. 7, 1923, p. 42-44. Sull'imitazioni dei piccoli detagli dei Microsporidi col fluosilicato di calcio. *Rend. accad. sci. Lincei,* 1925, p. 639-643, figs.

Silica exists in diastases (Matignon, *Compt. rend.,* Feb. 1, 1924) and shows diastasic activity (A. Mary and others). Bachmann has observed the production of pseudo-animated forms by evaporation of a silicic gel im-

Fig. 6.—Artefacts (× 153), produced by dropping solid CaCl₂ into a mixture of egg white and sodium silicate, containing some KF.HF.

Fig. 7.—Artefact produced by slow diffusion of CaCl₂ into a solution of sodium silicate containing a mixture of various salts, including KF.HF.

pregnated with benzene, *Z. anorg. Chem.*, **73**, 165 (1911). *See* Herrera, "Notions de Biologie et Plasmogénie," W. Junk, Berlin, 1906. The author believes that there are no imitations of living beings without silica. Cells of calcium fluorosilicate can be washed, fixed. stained and mounted in Canada balsam like natural cells, and the author has sent hundreds of these preparations to European laboratories. Silica is an oxidation catalyser [Shibata and Kaneko, *J. Chem. Soc. Japan*, **45**, 155, 78 (1924)], has therapeutic and nutritive properties and has been employed as manure by Oberlin, Lemmermann and many others. There are a great number of organogels and organic compounds of silica that may be the basis of a new chemical world.

Mercury. The author has published many works on the Beilstein amoeba (*Bol. Dirección Estudios Biológicos*, **1**, 211-254). Hydrosomes and mercurisomes. Pseudo-artificial beings in movement. Irised films. Influence of silica. Microscopical results. Imitation of Infusoria. (*Ibid.*, **2**, 40, fig.)

Mercury and aluminium. Conical growths in moist air, etc. (*Bol. Dirección Estudios Biológicos*, México, **2**, 40, fig.).*

Hydrochloric acid and sodium silicate. Imitation of fungi, *Puccinia, Exoascus*, Diatomaceae, *Pseudopeziza*, etc. Microscopic, hard. Infiltrations of the acid into the silicate. Cause: imperfect crystallisation of sodium chloride in silica. Stained and mounted in Canada balsam. *Mém. Soc. Sci. Antonio Alzate*, **26**, 1-49, figs. *Arch. Plasmologie Générale*, Bruxelles. Lamertin, **1**, Fasc. I, p. 21, pl.

Potassium chloride and silico-carbonate of potassium and calcium chloride. Crystallisation and, some time after, cellular forms. Amoeba. *See* Castellanos, "La Plasmogenia," Habana, 1921, p. 142.

Potassium silico-carbonate and calcium chloride. Reversible production of crystals and cells. "Protobios," 1917. Castellanos, *ibid.*, p. 141.

Potassium silico-carbonate in a porcelain candle, placed in a solution of calcium chloride. Nucleated cells in division. *J. Lab. Clin. Med.*, **4**, 479.

Infiltrations of calcium chloride in a solution of silicate and fluoride of potassium, in capillary compressed films, at 90° C. Complete cells with nucleus and filaments. Mounted and stained in slides. Many years of work and many publications. *Compt. rend.*, *loc. cit.* "Biología y Plasmogenia." México, p. 266-305. (*See* Figs. 3 and 4.)

Calcium carbonate in silica and silicates, and in colloidal silica. Spheroliths, cells, amoeba. Albumin in Harting experiments *is not necessary.*

Mémoires Soc. Alzate, **26**, 277-279, figs. "Comité de la Alianza Científica Universal," México, **I**, p. 19-21. The silica of reagents and albumin explains the forms and results of Rainey, Harting, Burke, Dubois, Kuckuck, etc. In all imitations of life silica exists and must be sought for.

Silicates and manganese salts. Artificial oxidases. *Mém. Soc. Alzate*, **29**, 331. Pseudo-plants of Traube prepared with manganese silicate decompose actively H_2O_2.

Colloidal silica and carbonates or sodium and potassium. Evaporation of the solution. Cells and imitation of human embryos. Cause: imperfect crystallisation of sodium carbonate in hygroscopic potassium carbonate, water and colloidal silica. *Terapéutica Moderna*, México, **31**, No. 2, p. 6. *See* also: Mary, "Dictionnaire de Biologie physiciste" (Paris), p. 67-69, figs.

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* See also paper by H Wislicenus, Vol. 1, this series. *J. A.*

VARIOUS IMITATIONS

Colloidal membrane pressure or hymenopiesis. Bol. Dirección Estudios Biológicos, México, **I,** 228-229, figs. 231-232, 235-236. Collodion dried on mercury; expulsion of metallic drops.

*Imitation of plants, buds, leafs, glands, bowels, brains of animals and of*
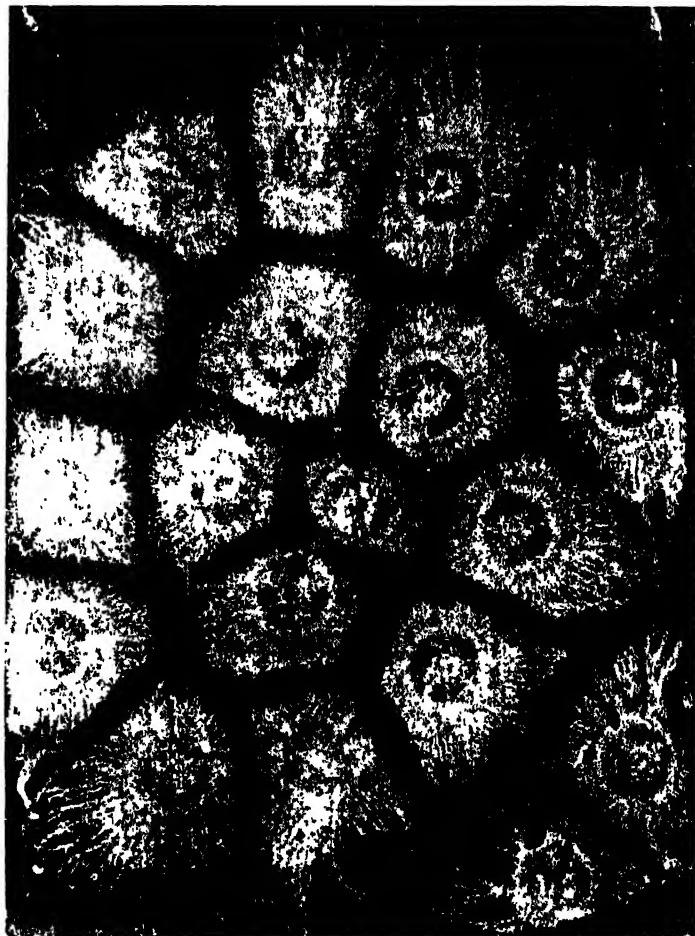
FIG. 8.—Imitation of Neurones: Alcohol dropped on sodium silicate films containing lamp-black, etc.

man by plastic solid matters injected in skulls or compressed in linen or screens. Bol. Dirección Estudios Biológicos, **I,** 341-366, figs. Castellanos, "La Plasmogenia," p. 33-39, figs.

Alcohol dropped in stained silicate films. Imitation of cells, nucleus, chromosomes, neurones, etc. *Bol. Plasmogenia,* Habana, **I,** 93-94, figs. *Homo,* Bruxelles, **I,** p. 80-82, fig. *L'Écho Mexique,* Paris, p. 11-12. *Rend. accad. sci. Lincei,* May 6, 1923, p. 508-510, figs.

Vibrations of lycopods in a membrane, forms of cells and infusoria. Bol. Dirección Estudios Biológicos, México, **2**, 45-62, figs.

Imitation of rhythmic coloration in insects, birds, cells, tissues, muscles, ova, glands, nucleus, starch, etc., by means of calcium fluorosilicate. Explained by sedimentation. "Semana Médica," Buenos Aires, September 21, 1922, p. 615-621, figs.

Imitation of Brownian movement. Drops of caustic soda (14% solution) in gasoline and olive oil (50%) from a height of 60-80 centimeters. Biological theory of the Brownian movement. "La Vie Universelle," *Bull. Assoc. Internat. Biocosmique*, **I**, no. 1, p. 13. *Mém. Soc. Sci. Antonio Alzate*, **32**, 209-211; *La Terapéutica Moderna*, México, Vol. **23**, no. 9, p. 65-68, fig. *Semana Médica* de Buenos Aires, Feb. 6, 1913, p. 305-308; May 28, 1924, p. 1227-1238, figs. These researches explain the granulations of colloids by a biological theory, and the general property of Brownian movement in colloidal matter. The experiment with drops of solution of soda is striking and should be repeated in every laboratory.

FUTURE WORK. SYNTHESIS OF THE PROTOPLASM WITH AMINO-ACIDS

According to modern ideas protoplasm consists mainly of proteins, nucleo-proteins, lipoids, water and salts. The production of a protoplasm endowed with organisation is a formidable task. The work of d'Herelle [*] and others on *bacteriophage* indicates how great is the complexity even of a bacterium. We here approach the border-line between "animate" and "inanimate matter."

Photosynthesis may have produced initial substances by reduction of CO_2, and also amino-acids according to the work of Moore, Webster, Baly, D. Berthelot and Gaudechon, Baudisch, Allen and Church, etc. The author has lately obtained "colpoids" with drops of soda (14%) and glycine or amino-acetic acid in gasoline and olive oil, with some lability.

The early work of Butler-Burke and Dubois on radiobes indicates that as a silica gel can adsorb the most minute quantities of radio-active substances (Ebler and Fellner) perhaps we shall prepare the Protobies, or initial beings, by means of a mineral nutritive solution, uranium salts or radium salts, and silicates and fluorosilicates. Radium may have furnished the primeval energy of the life, before photosynthesis determined by chlorophyll, which could not precede life.[8]

"It is known that the rays from radium have the power to stimulate all forms of life, even to the extent of speeding up to the growth of plants and of making dormant plants burst into bud. . . . It is because of its property of emitting negative electrons (beta rays), that potassium is a necessary constituent of all living matter. It may, however, be replaced under certain conditions, by other radio-active substances. Certain of the rays decompose ammonia, and water under their influence is subjected to electrolysis, yielding oxygen and hydrogen." [9]

[*] See paper by F. d'Herelle, this volume. J. A

[8] Maynard Shipley, "Wonders of Radium." Little Blue Book No. 1000. Haldeman-Julius, Girard, Kansas.

[9] See S. C. Lind, D. C. Bardwell and J. H. Perry, "The Chemical Action of Gaseous Ions Produced by Alpha Particles." VII Unsaturated Carbon Compounds." J. Am. Chem. Soc., **48**, 1556-75, 2335 (1926). See also S. C. Lind, "The Chemical Effects of Alpha Particles and Electrons," The Chemical Catalog Company, New York, **1926**, pp. 1-180, Carleton Ellis and Alfred A. Wells, "Ultraviolet Rays," The Chemical Catalog Company, New York, **1926**, illustrated.

Colloids and X-rays *

By Sir William Bragg,

The Royal Institution, London

It has been intimated to me that my contribution to the discussion of this question is to be concerned with the possible assistance that the new methods of X-ray analysis may give in the enquiry as to the nature and properties of colloids. It may be said, I think, that the new methods are only in their infancy; so that my account, besides being subject to obvious deficiencies for which I must not waste time in apologising, cannot be regarded as more than temporary and provisional. In a few years time the energetic research which is going on everywhere will doubtless have thrown much new light upon the matter, bringing facts into view which are now unsuspected and altering the relative importance of those of which we are already aware.

It will be convenient if I begin by setting out five points in respect to which knowledge of the colloids is to be desired, such knowledge as the X-ray analysis may be expected to contribute. We shall be in a fair way to explain the properties of the colloidal particle when we know not only its composition but in addition:

1. The internal structure of the particle; whether or no there is any periodic arrangement, and if so the pattern of that arrangement; also, possibly, the closeness with which the pattern is fulfilled.
2. The nature of the exterior layers of the particle, whether there is arrangement here also and if so how it is adjusted to the arrangement, if any, of the interior.
3. The average size of the particles, and the distribution of size about the average.
4. The general form of the particles, whether more or less spherical, or hair like or flaky or having any other pronounced characteristics.
5. The extent of any tendencies to orientation among the colloidal particles.

Let us remember at the outset that the new methods rest wholly on the existence of ordered arrangement within the bodies under examination. If there is no order, nothing can be done. But the limitation is not so severe as it might at first appear to be; for as enquiry goes forward, the really amorphous substance, that which cannot be described in terms of any lengths, recedes continually into the distance. Even a liquid has something for the X-rays to catch hold of. When a pencil of homogeneous X-rays is made to traverse a liquid stratum and afterwards to fall upon a photographic plate, the plate shows not only a central spot where the pencil struck it, but a diffuse ring surrounding the spot. The ring is a diffractive effect. The rays have a certain wave length, and the particles of liquid have at least one important linear definition, namely, their distance of closest approach. The co-existence of these two lengths must affect the scattering which obviously exists, and since the effect is defined by the angle through which the rays are deflected in various orientations, this angle must be a function of the ratio of the lengths.

There is no other way by which the mutual relations of the two lengths can manifest itself except as a ratio of no dimensions, such as the scattering angle. There may of course be other periodicities in the liquid, but this one alone must produce a ring. A number of ring photographs of this kind are shown in a paper by Sogani (*Indian Journal of Physics,* 1, 357). They differ considerably from one another; for example, the ring for cyclohexane is much more distinct and sharply bounded than that for nitrobenzene. Some of the rings are clearly double. Such variations can readily be explained as due to the irregular form of the molecule, the degree of exactness of the periodicity and so on. It is in fact difficult to assign the effects to the right causes since the latter are numerous and their influences are not easy to calculate. (See for example a paper by A. L. Patterson, appearing in a current number of the *Zeitschrift für Kristallographie.*) It may be that the molecules of the liquid are continually forming small groups, in an association which is more important and less temporary than a mere encounter between two molecules. It may be that there is periodicity in the arrangement of the atoms of which the molecule is composed. And again there may be assemblages of molecules so complete as to deserve the name of crystals.*

At the other end of the scale is the perfect crystal. Its effects upon X-rays that traverse it are of course far more complicated, more precise and more informative than anything to be obtained from a liquid that gives only one diffuse ring. It is now well known that the effects can be used to calculate the size and form of the unit of pattern within the crystal, and to determine the details of arrangement. In hundreds of simple cases we have been able to place the individual atoms, as for example in rock salt or aluminium. The greater number of crystals do not yield so readily because the problem is too complicated for our present experience; in particular the organic substances with which colloidal properties are so often associated, are very difficult to solve completely. Progress is made, nevertheless, with these latter substances, and in some cases such as that of the long chain compounds, very considerable progress. The yield of enquiry is of immense extent, and we may have to advance long distances in directions, which we hardly recognize as yet, before realizing our full capacity of contributing to the theory of colloids.

How this growing power to determine crystal structure will help us to find the internal structure of the colloidal particle is the first of the five queries which I have set down.

The X-rays can form a diffraction picture when passed through the colloidal preparation. This may be so simple that the actual structure can be deduced from the picture, as in the case of colloidal gold first investigated by Debye and Scherrer it appeared that the particles had the ordinary crystalline arrangement of gold, that of the face centered cube, one of the two ways in which spheres may be packed most closely together.

Or, it may be possible, if the details of such a picture are too scanty for the determination of the structure in full, to obtain at least some items of information which may be important Cellulose and stretched rubber will serve as instances in which this has been done.

And yet again we can surely anticipate that in the future we shall have learnt so much about the rules of arrangement of the atoms in the crystal that we may be able to sketch a probable design, requiring only some confirmation more or less from that which the actual X-ray picture gives. We

* See paper on mesomorphic states of matter in Vol. I of this series J. L.

might even hope to be able when given the composition to lay down the only possible design. Such powers will only be acquired by a sufficiently extensive study of crystals of all kinds, beginning with those which can. be obtained in sufficient size and perfection. We are of course very far from our goal as yet; there is an immense field to be explored before we can sort out the complexities therein. Only here and there we get a glimmering of some great law or rule which governs the building of material structure, and when we do so we find ourselves able to make the most interesting and unexpected generalizations. As an example let us take the discovery of the part played by oxygen in the formation of the silicates, and certain other compounds (W. L. Bragg and collaborators). It turns out that the oxygen atoms are in simple close packing in many cases, and as near as they can get to it in others; and that the other atoms, silicon, aluminium, iron and so on, are placed in the interstices of such a packing. Once such an idea is grasped it becomes possible to attack a new structure in which the same arrangement is likely to recur.. Or again in the realm of organic substances the discovery of the curious and exact periodicity in certain long chain compounds helps materially in beginning the examination of similar structures. But we may hope before long to get far past such elementary stages.

At present for example the X-rays have told us that in cellulose structure there is a certain unit of pattern containing four molecules, each having the composition $C_6H_{10}O_5$; and the unit is rectangular, its dimensions being $7.9 \times 8.45 \times 10.2$ Å.U. (Herzog and Jancke.) It is argued that this periodicity is contained within a larger unit. (Staundinger, *Zeit. f. Phys. Chem.*, 1927.) We have learnt a little about the mutual arrangement of the four molecules. The time will surely come when we shall know the place taken by each atom in the pattern. It may be that some one will one day hasten the solution by producing a single cellulose crystal; or it may be that before this is. done our knowledge of organic crystals will have advanced so far that the details already known will be enough foundation on which to build our calculations of the structure by aid of rules which we shall have discovered.

This then is the first point of attack of the X-ray methods on the colloid question. We are able to discover when any periodicity exists in the arrangement of the interior of the particle, and we hope to be able, as we improve in experience and technique, to complete our powers of discovering the details of the pattern.

The determination of the structure of a crystal particle, one of a great crowd, has its value of course; and the more that is learnt of the connection between the structure of crystals and their properties the greater will be that value. Yet even if the particle is crystalline in the main, its reactions with surrounding bodies depend on the arrangement of the atoms on its surface layers; and probably the effective skin is extremely thin, sometimes even monomolecular. The arrangement of the outer layers is affected both by the outside conditions and the internal constitution. It is the bridge between two regions. The surface forces determine the body's reactions, and they are related to a surface arrangement which most likely is not the same as that of the more ordered state within. How can the X-ray studies help us with that?

Not directly of course. The surface conditions are in general too shallow, and the layers are too few in number to give observable diffraction effects. There are, for example, no lines due to surface conditions in the

spectrum of colloidal gold. But I do not think we can overestimate the help we may expect to obtain indirectly from the X-ray studies. The fact of fundamental importance illustrated over and over again in the X-ray work is that the molecule is a structure of definite size and form, having different properties in its different parts. Its effects depend on how it is held. If the molecules on the outside of a body lie one way, they will do one thing; if another way, they will do another. Such effects are shown by polar crystals for example. It is possible to blind the action of all the molecules on a crystal face by spreading over it a monomolecular layer of some substance that acts like the button on a foil. The same fact is shown by the varying rates of growth of a crystal in different directions, and in numerous other ways.* Devaux, for example, showed that when a fatty acid was allowed to cool slowly on the surface of water, at first warm, the mass when cool could be wetted on the side that had been in touch with the water and not on the side that had been in contact with air. It is natural to suppose that the molecules had turned carboxyl groups to the water and methyl groups to the air.

Now the X-ray analysis particularly concerns itself with the matter of arrangement. It may not be able to show directly the management in the one or two effective layers of the surface, but it attacks directly the question of arrangement of the molecules where a greater amount of periodicity is attained. When we know what the molecule is like we shall be able to infer the arrangement which it will probably follow even on the surface. We should, for example, be able to infer from the study of the long chain compounds the probable orientation and arrangement of the sodium oleate molecules at a water surface, even were there no indications from any other source.

This is our second point. The attack upon it is of course merged to some extent in the attack upon the first. The outside arrangement cannot be assumed to be the same as that within and the whole action is a function of the arrangement. But the general study of crystals may be relied on to tell us about both. We are here concerned with those forces, whatever their origin, which bind molecules together even when chemistry considers the atomic linkages to be complete and describes the molecule as "saturated." We have the chemical bonds to consider as well, but these other manifestations of atomic influence come into obvious prominence in the solid state.

We now come to our third point. The size of the particle has, as is well known, an important bearing on its properties. It cannot be the only determining factor even in the case of a single substance, because the surface arrangement of the molecules or atoms must also be important. G. L. Clark tells us for example (Applied X-rays), that of nickel catalysts prepared by him the most effective was not that which had the smallest particles, and therefore the largest surface.

Now it is possible to estimate the size of the individual crystal in a mass of such crystals by a special measurement on the X-ray photograph. The width of the line due to a single crystal of a linear dimension greater than about 10^{-6} cm. is dependent, within errors of measurement, only on the geometrical conditions of the experiment, such as the widths of the slits. The line is very "sharp." But when the crystals are exceedingly small the line becomes diffuse. A well-known calculation by Debye and Scherrer gave a formula which has often been used to obtain the size of the particle from

* See e.g., W. M. Ord, "The Effect of Colloids on Crystalline Form and Cohesion (London, 1879); also J. Alexander, "The Effects of Colloids on Crystallization," *Kolloid Z.*, 1909. J A.

the broadening of the line. The experiment is not easy because so many disturbing factors must be allowed for before the true effect due to the size of the particles can actually be observed. A great deal of effort has been spent, particularly on the Continent, in the endeavor to overcome the many difficulties of observation. It is one of the researches most eagerly pursued at the present time.

Next there is the form of the particle to be considered. Here also the X-rays are able to help us. In the first place, they confirm us in our estimate of the molecule as a thing of parts and of powers that vary from part to part; and as I have said they are showing us the actual details of these variations. They help us to realize more vividly the processes by which crystals grow more readily in certain directions than in others. But they do more; they actually show us the existence of abnormalities of form, and in cases where other means are less effective. They tell us, for example, that in a long chain compound, stearic acid will again serve as an example, the long molecules tend first to range themselves side by side so that the solidification begins by producing fine flakes which proceed to lay themselves on other flakes to form the crystal. The edges of the flakes are very active, fresh molecules are bound firmly thereto as the flakes grow. But the sides of the flakes are far less active and the flakes slide easily over one another. They do not even attach themselves in proper relation to one another in all cases. Cleavage occurs readily parallel to the face of the flake, and turning on that face is common. Sometimes there is even a more serious maladjustment as Muller found in his distorted crystals. Trillat [*Ann. Phys.*, **6**, 1 (1926)] found that palmitic acid when cooled quickly in an apparently amorphous form and examined by X-rays gave the rings which are due to the sideways spacings of the flakes, periodicities which would be present even in a flake consisting of one layer of unit cells. There was no appearance of the long spacing. But when the acid was allowed to solidify slowly on the surface of cooling water, the same experiment showed also the rings due to the long spacing. The flakes had now laid themselves on top of one another to form more complete crystals, and so produced the periodicity which previously was absent. Trillat ascribes the result largely to the orienting effect of the water. This may be the only cause or it may be that the time of cooling is also a determining factor. The experiment helps us to realize how in the process by which the molecules address themselves to end up in solidification; the aggregates may take up special, if more or less, temporary forms. The subject has been dealt with at length by Friedel, who describes varying stages of the passage from the liquid to the solid state as smectic, nematic and so on.*

By such considerations G. L. Clark explains differences in the properties of carbon blacks. Certain charcoals produce only broad diffuse bands on the photographic plate; these carbons are characterized by high absorption activity, a definite solubility in oxidation media and a definite specific electric resistance. As they are subjected to heat treatment these characteristics all decrease in magnitude and though the X-ray diagram shows no change at first, the implication appears to be that the early stages of the physical development are too small for easy detection. As the process proceeds the X-rays begin to show what is happening. To use the author's words, at first "the atoms are becoming linked by valencies, but the layers so arranged are still too few or are so distorted and bent that graphite interference lines are impossible. The

* See Friedel's paper in Vol 1 of this series

amorphous ring, however, tends to become more sharply defined; other bands close to the center begin to appear very faintly, as the heating is increased; next, outlying bands, corresponding to graphite interferences which have been so diffuse as not to appear visible, define themselves, and then, continuously but very rapidly the entire graphite spectrum appears."

Thus there are various stages in the assemblage, marked by changes in the properties of the substance. The changes can be followed by means of the X-rays and interpreted in the light of the previous determination of graphite structure by the same means.

Lastly there is the question of orientation. This also has been studied extensively, and most interesting facts have come to light. A vast number of substances, fibers of all sorts, structure of living organisms, bones, muscles and the like, metals under strain, contain assemblages of minute crystals and these are found to be more or less oriented in relation to the direction of growth or strain. A drop of wax allowed to fall upon a plate and solidify there gives a diffraction pattern of continuous rings. A touch of the finger alters the pattern because the pressure orients the molecules in the formation of crystals and the pattern shows at once by its alteration the nature of the change that has occurred. Such effects have been studied most widely in connection with the deformation of metals under heat and stress, because the application to important industrial questions is so important. But it is clear that a field is opening up in which are to be studied the processes of growth and strain in substances usually called colloidal. The enquiry meets with many difficulties as yet, but the interest of the question and the rewards of a successful attack are sufficient inducement to continuous and eager research. As an example we may take the very interesting investigation of Katz, Hauser and others on rubber. Nothing is more curious than the sudden appearance of crystallization in rubber when it is stretched and the equally immediate disappearance of the crystallization when the strain is removed.

The X-rays then can find something to measure even in the substances that most seem to deserve the title of amorphous. Through all the grades of increasing periodicity, assemblages of molecules even in temporary partnership, groupings where only some out of several modes of welding have been at work so that the resulting mass has not achieved its final form, arrangements forced on the molecules from without and not brought about by their own mutual action as is stretched rubber, orientations brought about by contact with other substances as when fatty acids are arranged by contact with a glass surface from which the last film of moisture has been removed, through all these partial crystallizations up to the complete periodicity of the single crystal where the X-rays have their fullest opportunities, it is now possible to follow, if haltingly, all the way. And it is because the great range of properties which we associate with the colloidal state are connected fundamentally with the arrangements and periodicities that reveal themselves to the X-rays that we may hope by their aid to obtain a better understanding of the colloid.

The Ageing of Colloids

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A fundamental characteristic of colloids is that they do not form *stable* systems; the particles dispersed through the system (the micells), upon which their colloidal properties depend, are chemically unstable complexes (adsorption complexes), in which there take place continuous transformations induced by heat, by light, by electric fields, by electronic discharges, by ions, or by the molecules of the dispersion medium. On this account, there result variations in the properties of the system, by which the dispersed phase tends to lose its characteristic properties, and the colloid begins to coagulate, reaching a stable state when coagulation * has become complete. This process of transformation, taking place in the properties that define the colloidal state, which is a consequence of the incessant evolution that these systems undergo and which terminates in coagulation, is what we call "ageing" of the colloid. We shall deal with the intensity of these physico-chemical variations as indicated by appropriate methods of measurement.

According to the physical concept, the dispersed particles in colloidal systems constitute entities which persist within certain limits of variability of the factors of equilibrium of the system, and which, having an electric charge, resist the loss of their individuality. This tendency does not signify chemical stability, for the adsorption complex which constitutes the micell changes as long as the colloidal state persists. We can follow the process of evolution which determines the ageing of the colloid by studying, where possible, the variations in color, the structure of the particles, the variations in viscosity, in surface tension, in electric charge, in conductivity and in catalytic power. We shall give here a short résumé of those variations which we have studied in considering the different aspects of the question, for we do not wish to repeat here what we have already published elsewhere in original articles.

With red colloidal sols of gold prepared in a way which ensures great stability, it is easy to see how in a few days the red color changes to violet and finally to blue; during this process, the particle of solid gold which the red hydrosols possess ("protones", Zsigmondy) become transformed into "polyones", and this is a process of coagulation (ageing) which is restrained while the system is stabilized but is accelerated by ionic action or by electric fields.[1] In some other cases, e.g. in tungsten colloids [2] we have found analogous phenomena and the observation of these changes should always be carried out, if one wishes to make them under the best conditions, with the aid of the slit ultramicroscope of Zsigmondy.

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* Crystallization is the finally stable state, though it is often not reached in measurable time. *J. A.*
[1] Clavero, "Investigaciónes sobre el poder coagulante de los iones," *Trab. Lab. Inv. bioquim de Zaragoza*, Vol. I (1921).
[2] Clavero, "Estudios sobre coloides de tungsteno," *Trab. Lab. Inv. bioquim. Zaragoza*, Vol. II (1922).

The viscosity of colloid systems suffers variations of a different order and even of different sense, according as the system involved is or is not stabilized; if one works in the presence or absence of air; if there are present ions, etc.,[3] but in all these cases one observes that the viscosity is not constant. With aqueous solutions of gelatin (0.5%) one observes, in an interval of 5 hours, a variation in the viscosity from 16.5×10^{-4} to 17.8×10^{-4}. If the solution of gelatin is kept in a sealed tube and heated to 120° C., we find that the variations in viscosity are very much less.[4] In the hydrosols of palladium, obtained by the reduction of a palladium salt with hydrazine hydrate, the variations in the viscosity compared, for example, with those of gold sols, are less when the system is stabilized with lysalbinate of soda.

In the case of gelatin dispersed in water, we can interpret the variations in viscosity as changes of a chemical nature, which are brought about when the H and OH ions "catalyse" the hydrolysis of this protein (Davis, Brown, Oakes),[5] and in all these cases, according to our view, the cause of this variation and of others which we shall cite, is a chemical transformation from which will result the instability of the complex which constitutes the micell.

The physical individuality of the micell persists for a certain time because they are material particles provided with a certain electrical charge. This produces between them a mutual repulsion of electrostatic origin which opposes their agglutination or their separation from the medium in which they are dispersed. It is on account of the existence of this electrical charge that we are able to observe the phenomenon of migration when we establish an electric field in a colloidal system.

In electrosols of silver, my collaborators, Drs. Rius and Llanas, have made measurements in order to show the process of evolution of these colloids by studying their electric charge;[6] they have determined the mobility by a special arrangement which, according to the work of Dr. Rius, offers great exactness of method in determinations such as this, so easily nullified by various causes.[7]

We measure the electrical charge by means of the formula: $c = \dfrac{x}{nv}$, where c represents the electrical charge, x the specific conductivity of the colloidal solution, n the number of micells per cm.[3] of the colloid and v the *cataphoretic* velocity. In a large number of these studies we have been able to observe that the colloids soon after their preparation tend to increase their faradic charge, and this increase is followed, or not, by a diminution, according as the colloid changes rapidly or slowly.

Of all the variables which determine the electric charge of the micell, the conductivity is that which varies within the widest limits; as a consequence the measure of the conductivity of the system and of its variation, gives us the easiest method of following the process of ageing which gives rise to variations in the electrical charge of the micell.

Aqueous dispersions are capable of being ionised; they constitute systems (ionic solutoids), the electric charges of which vary with the substance dispersed, the dilution, the temperature; but supposing these factors constant,

[3] Turmo, "Investigaciones sobre la vejez de los sueros," *Revista de los Laboratorios Ibero Americanos*, Barcelona (1924)
[4] Rocasolano, "Physikalische-chemische Hypothese uber das Altern," Traduc Dr. Thiessen, *Kolloid chem. Beihefte*, **19**, 12 (1924)
[5] Davis, Browne and Oakes, *J. Am. Chem. Soc* (1921)
[6] Rius and Llanas, "Sobre las variaciones de carga eléctrica en los electrosoles de plata," *Trab Lab. Inv. bioquim. Zaragoza*, Vol III (1923).
[7] Rius, "Sobre la cataforesis eléctrica y su medida," *Trab Lab. Inv. bioquim Zaragoza*, Vol III (1923).

the electrical charge and the conductivity should remain constant. In colloidal systems things do not turn out this way; their conductivity, their electric charge, their degree of dispersion or number of particles per unit volume, vary incessantly, for what varies in them is the chemical constitution of the particle which is the origin of these variations whose development gives place to processes of ageing of the colloids.

This evolutionary process of which we have just been speaking, takes place through a mechanism and with an intensity varying according to the nature of the dispersed substance. When we are dealing with metallic colloids, which are easily ionised, the conductivity of the system increases because of the ions which separate from the micell and cause this effect.

We have been able to verify this in the case of silver sols, the micellar change of which varied in a period of 90 days from 0.03×10^{-13} to 8.45×10^{-13} coulombs. With these colloids, and metallic electrosols in general, with similar conditions of medium, dispersion, temperature, and time, the variations of the electric charge depend on the intensity of the current which produces the sol.

There results from this study the fact that those electrosols which are regarded as the most stable (that is to say, those produced with a current of from 2.4 to 4.0 amperes) present, as a consequence of less rapid evolution, the smallest variations in electric micellar charge and electrical conductivity. Table I gives some examples.

TABLE 1.

Current Used to Produce the Electrosol		Mg. of Silver Dispersed Per Cent	Conductivity			Electric Charge of the Micel, in Coulombs		
Amp.	Volts		Initial	Days	Final	Initial	Days	Final
2.0	106	39	266.9×10 '	17	807.9×10^{-7}	10.9×10^{-11}	17	101.0×10^{-13}
2.4	90	5	52.0 "	85	249.2 "	3.65 "	85	51.6 "
2.9	109	5	48.3 "	90	212.0 "	0.93 "	90	8.4 "
3.5	100	7	61.6 "	66	173.0 "	1.39 "	66	5.8 "
4.0	103	8	80.6 "	18	173.0 "	0.68 "	18	3.4 "
7.0	104	18	145.5 "	32	1730.0 "	8.81 "	32	37.9 "
12.0	98	19	.. ...	..	. ....	0.70 "	23	104.8 "

When we deal with organic colloids, these variations are quite different. These substances are less apt to produce ions; there results a diminution in conductivity, and when this attains a limiting value, peculiar to each substance, the colloid coagulates. We can cite as an example of this type of variation, those which one can observe in the case of calves' serum, of which the conductivity when freshly obtained is 86.7×10^{-5}; at the end of 48 hrs. this decreases to 68.7×10^{-5} and at the end of 72 hours, when coagulation occurs, it has become 58.8×10^{-5}. It may be remarked that these observations were made under *aseptic* conditions but in contact with air, while, if one keeps the material in sealed tubes or flasks, the variations in conductivity are much less pronounced. This fact recalls an analogous observation which we have already pointed out in speaking of variations of viscosity, and it explains the fact that the properties of sera derived from the biochemical activity of their colloids, persist a great length of time when the substances are kept out of contact with air.

It is incontestable that, among all the interesting properties of colloids, their catalytic property is of the greatest importance. In many industries catalytic substances are colloidal while in the totality of biochemical reactions, which are produced in living organisms, the catalysers are in the colloid state. It follows that of all the variations of properties which colloids undergo during their process of evolution, which is their ageing, those with respect to their catalytic power are the most important.

Our studies on the catalysing action of electrosols of platinum and hydrosols of palladium in the decomposition of oxygenated water, led us to conclude that the catalytic power of these systems increases with the age of the colloid for the first few days from the time that they are prepared, and, afterward, diminishes, not in a uniform manner but with irregular variations. In several papers, we have published results obtained from various series of experiments.[*] Figure 1 represents graphically the case of an electrosol of platinum (of 0.0026%) for which we have followed the variations in catalytic power for
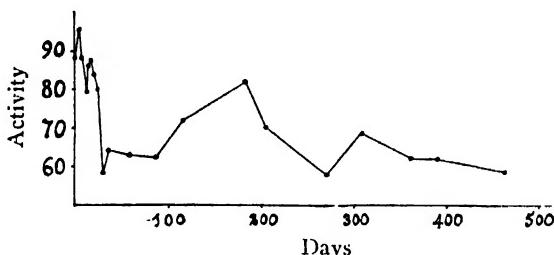
FIG. 1.—Variation of catalytic power of a platinum hydrosol on ageing 450 days.

450 days. The figure represents the quantity of oxygenated water decomposed at the end of 48 hrs. catalytic reaction at a temperature of 35°.

We have generalized these observations by the study of the catalytic power of diastase which catalyses the reaction of the inversion of saccharose (invertase). Working under the conditions of asepsis necessary to prevent infection of the diastasic liquid, we have found variations in the catalytic power of the same order as those observations on electrosols and metallic hydrosols.

In this case, as in those studied previously using metallic colloids as catalysers, the catalytic power of the diastase liquid grows to a maximum and then decreases in consequence of the evolutionary process which colloidal catalysers suffer; this is the ageing of the diastase which can be observed in many other cases with oxidases or with hydrolases.

If we work with stabilised colloid for the study of these evolutionary phenomena which constitute the ageing of colloids, we observe analogous variations, but they take place at much slower rates, as if the stabiliser acts as an inhibiter of the evolutionary process. In particular, the variations in color and micellar structure are very much slower, the variations in viscosity and surface tension much smaller, while the measures of electric charges on the system leads one to conclude that because of the presence of the

[*] Rocasolano, "Sur le vieillessement des catalyseurs colloïdaux." "Variations du pouvoir cathalytique dans les electroplatinsols," *Compt. rend.* (1920 and 1921).
"Estudios sobre la evolución de los sistemas coloidales," *Trab. Lab. Inv. bioquim. Zaragoza*, Vol. I (1921).

stabiliser, the micellular electric charge and the micellular conductivity increase.*

The catalytic power diminishes on the contrary and this experimental fact and another of the same order, which we have found,[9] suggest the idea that it is necessary to place the study of colloids as catalysers in the domain of chemistry, for the catalyser is transformed in so far as its catalytic action is concerned, and if it is stabilised its transformation is slower and in consequence its catalytic power is less.

Stabilized colloids are then less active catalysers than those same colloids unstabilized; their variations of catalytic power are slower because the evolutionary process, which is the ageing of the system, is retarded by the presence of the stabilizer.

These variations of catalytic power, which we present as characteristics of the ageing of colloids, should be observable also in the colloids which constitute the living plasmas, and passing from the simple systems observed to the complex systems which constitute living matter, we have verified analogous phenomena. Leaving aside many determinations made on the fermentative power of pure cultures of *saccharomyces ellipsoidus* both old and young, which confirm these ideas, we shall now report some quantitative determinations of the activity which we have found in old and young cultures of *bacillus radiciola,* used as a fixer of atmospheric nitrogen.

Old pure cultures of the bacterium used to fix nitrogen, which may be extracted from the nodules of cultivated red clover in proper bouillon (infusion of beans, "manitée") fix much less nitrogen for the

Fig. 2.—Variation in nitrogen-fixing power of *bacillus radiciola* with time. A = a fresh culture: B = an old culture.

same time, same quantity of bouillon, same surface and temperature, than do the young (fresh) cultures of the same microörganism. Figure 2 represents graphically (A, the fresh culture, B the old culture) the variations which express the ageing of the microörganism defined by the enfeebling of the catalytic power of their diastases which are the catalysers produced by the cellular activity. In the experiments which Figure 2 represents, we studied the catalytic action of graphite in the fixing of atmospheric nitrogen by the bacillus radicicola; this shows how the old cultures (graph B) fix less nitrogen in a given time and under similar conditions of temperature and medium, than do fresh cultures. In these graphs, the abscissæ give the quantity of graphite present (in centigrams) and the ordinates the nitrogen (in milligms.) fixed at the end of 20 days from inoculation.

According to these ideas, increasing the virulence of a culture is only a

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* The importance of colloidal protection (stabilization) in the organism is obvious. See paper by J. Alexander in Vol. I of this series, p. 619. *J. A*
[9] Rocasolano, "Sobre el poder catalítico de los sistemas coloidales," Libro Homenaje al Dr. Cajal, Madrid, 1922, part 2.
"Estudios sobre fermentos metálicos," *Revista Universidad Zaragoza,* Vol. I, No. 1 (1924).

process of rejuvenescence accomplished by means of well-known procedures used to this end.

The maturing of some stains used in histology is nothing but a process of ageing which takes place in the colloids of which they consist, and the acceleration of the ripening of the color by oxidation is simply speeding up the ageing process.

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If we wish to investigate vital processes, we must emerge from the field of morphology, for a multitude of experimental facts prove that life does not reside in the architecture of the organism. The continuous series of transformations that characterize vital phenomena, come into being when heterogeneous substances meet, such as those which form the cytoplasm and the nucleus, which are so constituted that they produce, in each other's presence, transformations of matter accompanied by energy changes. All this gives rise to physico-chemical processes which lie at the basis of physiological activity.

All the components of living matter are of interest, because physiological processes take place in them all; but most important are those components in the colloidal state—that is quite evident. So intimate is the connection between the colloidal condition of living plasmas and life itself, that, if for any reason whatever, the cellular colloids lose their stability, life ceases. We draw this conclusion from our studies on the Brownian movement in cells.[10]

Considering vital phenomena in their physico-chemical aspect, we see that there is an intimate relation between the characteristic properties of a colloidal system and the way the colloids evolve toward coagulation by means of physico-chemical changes which determine the nature of their ageing. We have advanced a *physico-chemical theory of ageing*, by which we attempt to interpret the phenomena of degeneration which constitute the ageing of organisms on the basis of our experiments on the ageing of colloids.

Each colloidal system exhibits different processes of transformation, based on the nature of the chemical changes that their micells undergo. We have already referred to the case of silver, and with electrosols of platinum we believe that variations in the concentration of oxygen alter the composition of the micell and thus reduce its catalytic activity. The evolution of the important cellular colloids, which are mainly proteins, is brought about by changing their degree of *hydrophility* which tends to diminish; therefore the development of ageing is intimately associated with a process of dehydration.

Dehydration of the protein micell produces changes in surface tension and viscosity.*

There is, besides, a change in the electric charge of the particles, and consequently also a change in the equilibrium factors of the system. The catalytic power of catalyzers, due to the micells, diminishes; and the sum total of these changes affects *metabolic* processes, leading to what we call ageing. It may be that the degeneration of the glands of internal secretion is the result of, and not the cause of ageing, whose origin, instead of being due to the degeneration of this or that organ, should more logically be sought in the changes of the basic constituents of the living plasms, due to the evolution of their constituent colloids.

* See paper by J. Alexander, "The Zone of Maximum Colloidality," *J. Am. Chem. Soc.*, **43**, 434 (1921); also Chapter I in Vol. I of this series. *J. A.*
[10] Rocasolano, "Sobre el movimiento browniano," *Trabajos de la Sociedad de Biologia, Barcelona* (1916).

Hydration and Viscosity of Sols in the Presence of Electrolytes

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Chemists in dividing colloids into two groups, e.g., hydrophile and hydrophobe, have made this tacit assumption that the hydrophobe colloids are readily precipitated and do not form gelatinous precipitates when coagulated; whilst hydrophile colloids are assumed to be precipitated with difficulty and give gelatinous precipitates when coagulated. Moreover it is also assumed that the hydrophobe colloids are not reversible whilst the hydrophile colloids are generally reversible; the former are precipitated by low concentrations of electrolytes whilst the latter only by high concentrations of electrolytes. There is also the belief in the minds of colloid chemists that hydrophile colloids are much hydrated whilst hydrophobe colloids are not hydrated at all or only feebly hydrated. We are of the opinion that no direct experiments have yet been made to prove the above conclusion, which is based only on some indirect evidence. Usually hydrophile substances show the property of swelling when the dry jelly is put in water. The dry materials constituting the hydrophobe colloids do not show the property of swelling which will be discussed later on. Moreover the viscosities of the hydrophile colloids are much greater than that of the medium, whilst the viscosities of the hydrophobe colloids are practically those of the solvents. Facts of this nature have been explained on the hypothesis that substances like gelatin, protein, albumin, etc., have more affinity for water than colloidal gold, silver, etc. Hofmeister and his colleagues have proved that substances of the former class are only precipitated by high concentration of salts whilst those of the latter class are coagulated by low concentrations of salts. In the subsequent pages it will be shown that Hofmeister and Pauli believed that these differences in the behavior of the two classes of colloids towards electrolytes can be explained on the hypothesis that substances like albumin, gelatin, etc., are more hydrated than gold, arsenious sulfide, etc., and that in order to precipitate the colloids, dehydration is necessary; and that greater quantities of salts which have also affinity for water will be necessary to coagulate hydrophile colloids than the amounts necessary to coagulate hydrophobe colloids.

We know that the question of the hydration of ions stands essentially on qualitative experiments and up till now we have only a general idea of the order of the amount of hydration of the ions, no really quantitative data on the amount of hydration are available.[1]

The question of the hydration of colloids stands on a worse footing, and even preliminary qualitative experiments have hardly been made; yet we can

get certain indirect evidence with regard to the state of hydration of colloids. It seems certain that colloid particles having a large surface are bound to adsorb molecules of the solvent to their surfaces. Moreover we know that the process of adsorption is partly chemical and specific in nature; consequently there is no doubt that some colloids would adsorb larger quantities of water than certain other colloids. Moreover there is always the possibility that certain colloids would adsorb water more firmly than other colloids. It seems quite possible that substances like gelatin, albumin, etc., can adsorb water more firmly and in larger quantities than sols of gold, silver, As_2S_3, etc.; but this has not yet been proved directly.

If a crystalloid is thrown into water, it subdivides in it until finally it is completely dissolved. The particles of salt or sugar lose their cohesion. A colloid in contact with water increases in volume, it swells; its particles retain their cohesion. This property, however, is possessed only by hydrophile colloids.

The imbibition of water, that is swelling, may either go on indefinitely as in the case of colloids so that finally the particles are torn asunder and a solution or sol is formed as in the case of albumin, or the imbibition may reach its limit very rapidly as in the case of wood. Between these there are all sorts of transitions, e.g., glue. In the organism, gels having very slight ability to swell, serve as covering and frame work (e.g., hide, shells, wood, etc.). They are intended to retain their outward form. The same is true for the supporting tissues of the individual organs and even of the cells, vessel walls, the membranes of the intestinal canal, connecting tissues, the vascular bundles of plants, cell membranes, etc. On the other hand the cell content possesses the ability to swell to a high degree.

Every organ has a definite normal fluid content. A healthy plant has a definite turgescence and the protoplasm of a healthy animal a given degree of swelling; every abnormal change in this, signifies illness or even death. Frogs may lose one fourth of their body weight upon drying, as has been shown by Overton.[2] Although they contain about eighty per cent water, the osmotic pressure of the blood is almost double. The explanation of this is, that only a portion is water of solution; the remainder is water of swelling. On drying the water of swelling is more strongly retained than the water of solution. Swelling exhibits manifestations of energy to a marked degree and many examples are given in Ostwald's "Grundriss," Bechhold's "Colloids in Biology and Medicine," as well as in Bancroft's "Applied Colloid Chemistry."

No colloid can exist in the organism without water, and small quantities of salts, because they condition the turgescence which is characteristic of living colloids. The earliest stages in the development of life are accompanied by powerful processes of swelling which soon reach a maximum, and then pass over into a shrinking, which becomes progressively greater, until death occurs. In the case of plants, the struggle for water between seed and soil starts with germination. Growing and full-grown plants show a certain turgor, i.e., a fullness or tension like a distended rubber balloon, while a dying plant is withered and poor in water. In human life old age is associated with decrease of water content; turgescence in general, and of the skin in particular is obviously lost. The organism taken as a whole, during its life evidently passes through the curves of swelling and shrinking of an

[2] *Biochem. Zentralbl.,* 2, 518.

inelastic gel. In individuals of the same species, the water content is fairly constant for the same period of life.

Quantitatively, the substance most important for the organism is water. Colloids and water are mixed up in the organisms and are usually associated together. An organism without water is lifeless. We can imagine such an intimate and varying relation to water only in a colloidal system. The process of swelling, the adsorption of water, and shrinking to complete dryness exhibit sudden changes in condition. When we consider a solution of a crystalloid in water we see something entirely new with very different properties, e.g., a solid crystalline matter, appears from a solution on losing water. Such a system would be unable to maintain correctly the constantly oscillating water balance and the normal condition of swelling in the organism, or to act as an accumulator of large quantities of water, like muscle, and release it for use when necessary. Such a system cannot stand the irregularities which the organism occasionally experiences as a result of physiological and pathological processes, and the organism because of its colloidal nature after adsorption constantly restores its normal state of swelling by means of secretion.

In all the processes of imbibition, it is important to remember that the total volume, gel plus water, is less after swelling, although the volume of the gel itself increases so much. In order to compress water to the extent implied in the total change of volume, a pressure of some 300 atmospheres is necessary, so that it is plain that heat must be evolved in the process of imbibition.

Hofmeister (1888) in fact. found the action of neutral salts on the process of imbibition to follow the same series as that of "salting out."

Samec[3] calls attention to the fact that parallel to the favoring effect exerted by the anions of Hofmeister series on the imbibition of water by starch. there runs a set of physico-chemical properties of the salt solutions themselves. These are, rate of diffusion and compressibility, which increase with the favoring action, while surface tension. internal friction, electrical conductivity, diminution of solubility of other solutes, maximum density, effect on catalysis of esters, inversion of cane sugar by acids, and dissociation of weak acids, are properties which decrease along with increase of favoring action. At first sight it would seem natural to connect these various phenomena with hydration of the respective crystalloids in solution. A part of the solution is held in this way in the region of the solute, so that any process in which water is concerned would pursue a different course in presence of crystalloids than in their absence, and in general, the change would be of the same kind as that caused by increase in concentration. There seems, however, to be some additional factor, because there are some crystalloids whose solutions produce more swelling than pure water does.

According to Zsigmondy,[4] the lowering of vapor pressure in the imbibition of water by silica gels is due to the formation of a concave meniscus, not to formation of hydrates. Imbibition is the filling of hollow spaces in this case, not the taking up of water into actual substance.

Gelatin is sometimes used to remove water from say 90 to 95 per cent alcohol. Bayliss[5] showed that it does remove water from 90 per cent alcohol, so that this becomes stronger, but no increase in volume of the gelatin was to be detected. In order to determine the volume after immersion in alcohol,

[3] *Kolloid chem. Brihefte*, **3**, 123 (1911).
[4] *Z. physik*, **14**, 1098 (1913).
[5] "Principles of physiology," p. 101, 1918.

the pieces were allowed to dry for about a minute in air; the liquid water evaporated from the surface passed off with the alcohol, a phenomenon that could not have taken place in so short a time if water had penetrated into the substance of the gelatin.

It will be interesting to note that substances like egg white, gelatin, casein, cellulose, starch, fibrin, which are proved to be amorphous by X-ray analysis of Debye and Scherrer [6] are the very substances which show the phenomenon of swelling. On the other hand, gold is a typical hydrophobe colloid and is proved to be crystalline by X-ray analysis; it is neither gelatinous nor shows the phenomenon of swelling. Gels of silicic acid and stannic acid are intermediate in properties between gelatin on the one hand and gold on the other.

Freundlich and Scholz [7] have shown that with gold sol and von Weimarn's sulfur sol the action of the precipitating cations showed an additive relationship, while the precipitating action was not additive with As_2S_3 sol and Oden's sulfur sol, particularly with mixtures of ions having widely varying precipitating powers that are said to be highly hydrated. Since von Weimarn's sulfur sol is not hydrated, while Oden's sol is hydrated, Freundlich and Scholz conclude that the hydration of the colloid and the precipitating ion is of primary importance in producing ionic antagonism and so in determining whether the precipitation values of the mixtures shall be additive or above the additive value. They are thus led to believe that As_2S_3 is a hydrophile sol although it is not usually so considered; and finally they suggest that the behavior of colloids with mixtures is a suitable means of determining to what extent the stability is influenced by hydration. "We believe with Neuscholz," say Freundlich and Scholz, "that the ionic antagonism observed in a test tube is closely related to the phenomenon which has been recognised as such in the biological action of electrolytes and has been followed by Loeb, Lillie, and others. As an example of this kind of biological action of electrolytes may be mentioned a series of investigations by Lillie.[8] The cilia of the larva of a ringworm *Arenicola*, are liquefied by a solution containing sodium ion; the addition of a small amount of a divalent cation stops this process. At the first glance, our observations seem to be different in certain respects from this kind of biological action of electrolytes, and from the ionic antagonism observed by Neuscholz." In both of the latter cases it was frequently observed that the action of the univalent cations could be nullified by the action of divalent cations while with sulfur sol the action of divalent cations was decreased under the influence of univalent cations. This is probably only a superficial difference. The coagulation of the sulfur sol is realized only at such concentrations of univalent cations that small concentrations of divalent cations cannot annul their influence by displacing their adsorption. If we had studied phenomena like the biological action of electrolytes which could be observed at smaller concentrations of univalent ions, we could have nullified their effect by divalent cations. According to the view arrived at in this paper, we believe that in these biological processes as well as in the coagulation of hydrophile sulfur sol, pure electric influences of pronounced ionic antagonism cannot be produced; for ionic antagonism a hydration influence is always necessary, such as appears with increasing strength when we go from

[6] *Z. physik*, 17, 277 (1916)
[7] *Kolloid chem. Beihefte*, 16, 267 (1922).
[8] *Am J physiol*, 10, 433 (1904).
[9] *Pflugers Arch.*, 17, 181 (1920).

gold sol or Weimarn sulfur sol to As_2S_3 sol and finally to hydrophile sulfur sol."

From this account it is evident that Freundlich and Scholz are convinced that the extent of hydration of a colloid is the most important factor in causing the ionic antagonism which results in precipitation values for certain mixtures of electrolytes that are considerably above the additive values.

The conclusions of Freundlich and Scholz would have been more convincing if they extended their observations to the sols of the hydrous oxides which are quite as representative of the class of hydrophile colloids as is colloidal sulfur.

On the other hand, the results of Weiser[10] are not in accord with the conclusions of Freundlich and Scholz that the hydration of a colloid and of the precipitating ions is of primary importance in producing ionic antagonism and that the behavior of a colloid with mixtures may furnish a suitable means of distinguishing a hydrophobe from a hydrophile colloid.

The result on the coagulation of sols by a mixture of electrolytes obtained in this laboratory, do not support the conclusion of Freundlich and Scholz.

It is well known that when ferric hydroxide is prepared by mixing an alkali and a ferric salt at the ordinary temperature and it is washed free from electrolytes, the color of the precipitate is brown and this hydroxide readily dissolves even in dilute HCl. When this freshly precipitated and well washed ferric hydroxide is kept in contact with water at the ordinary temperature, the color of the precipitate becomes deep red and after the lapse of about sixteen months we find that the chemical reactivity of the hydroxide is greatly decreased, as it does not readily dissolve even in concentrated HCl, and its adsorptive power is also greatly decreased. We have observed that arsenious acid, tartaric acid, etc., are not as readily adsorbed by this red hydroxide as by the brown hydroxide. More or less similar behavior is obtained with hydroxides of chromium, aluminium, zinc, etc. We are of the opinion that even in contact with water these hydroxides can lose their water and become converted into less hydrated and less active compounds even at the ordinary temperature. These changes are similar to those undergone by blue hydroxides of copper and cobalt.

It seems to us that this phenomenon is a perfectly general one and can follow from the general principle that the free energy of a system tends to decrease and is also a corollary of the LeChatelier-Braun principle. It is well known that when concentrated sols of $CaCl_2$ and $(NH_4)_2CO_3$ are mixed, a bulky gelatinous, readily soluble and reactive variety of $CaCO_3$ is obtained, and this variety resembles most the state of solution from which it is separated. In course of time even in contact with water this bulky variety passes into a less energetic, less soluble, less hydrated and compact form of $CaCO_3$. Exactly similar phenomenon takes place in the formation of many other precipitates.[11]

It should be emphasized that the adsorptive power of substances in the course of their formation is much greater than that of substances when once formed.[12] Recently Kleeberg[12a] has observed that a beryllium hydroxide precipitate completely loses its adsorptive power in 14 days.

[10] *J. phys. Chem.*, **28**, 232 (1924).
[11] Compare Willstätter and Kraut, *Ber.*
[12] *J. phys chem.*, **26**, 836 (1922).
[12a] *Kolloid Z.*, **37**, 17 (1925).

In this connection it is interesting to investigate the phenomenon of "Ageing" from the point of view of hydration of substances.

Though in the absence of chemical changes crystalloids retain their physical properties, in the case of colloids after the lapse of some time changes occur which are commonly called ageing. For instance, silicic acid which has been freshly prepared from water glass solution and HCl is at first dialysable but loses this property after a few days. Most of the ageing phenomena of sols are characterized by the fact that the particles of a highly dispersed solution gather together to form larger particles, that their sensitiveness to flocculation is increased or that they spontaneously coagulate. In the case of gels, their elasticity suffers change and they become optically inhomogeneous or turbid.

If solutions of hydrophobe colloids, e.g., As_2S_3, Au, etc., without protective colloids are permitted to stand for some time, they flocculate after a short time or else after the lapse of years.

We must emphasize that the changes in the colloidal system need not always consist in a diminution of the dispersion. Occasionally we find that the particles become smaller with the lapse of time, but this has hitherto been observed only in the case of hydrophile colloids (glycogen, benzopurpurin, hemoglobin, lecithin, etc.). This question will be taken up later on.

With ageing there occurs shrinking, which begins already with intra-uterine life. In the third month of human fetal life the water content is 94 per cent, at birth it is 69 to 66 per cent, in adult life 58 per cent. We may say in general that with ageing there is a decrease in the swelling capacity of the organ colloids. This holds both for animal organisms, which lose water as they grow older, and for plants.

We are convinced that in the phenomenon of ageing the dehydration of the colloid particles play a very important part. If we assume that the charge on the colloid particles due to ageing are not appreciably decreased, we have to assume that the chemical activity and surface of the particles are decreased.

McBain and Taylor [13] observed that, on salting out sodium palmitate with strong alkali, the concentration of the alkali in the mother liquor is greater than before, showing that water had been abstracted in forming the curd fibres. When calculating these results, they made the assumption that no appreciable quantities of sodium hydroxide are taken into the curd fibres themselves, and hence were able to calculate the hydration of the curd fibres, thus obtaining minimum values. The hydration varied from 3.4 mols of water to one of sodium palmitate in the presence of 3 N caustic soda to 6.5 mols of water to one of sodium palmitate in the presence of 1.5 N caustic soda. The stearate was less hydrated.

These results were followed up by experiments in which Martin salted out sodium palmitate with saturated sodium chloride in the presence of small amounts of sodium sulfate, which here served as reference substance. This curd was found to contain two mols of water to one of palmitate.

Salmon [14] made use of a wholly independent method, applicable, however, only to solutions saturated with one or more salts. His results proved that the composition of the curd fibres in the presence of saturated brine is 2.1 mols of water to one of sodium palmitate at 90° and 2.4 mols water at 50°.

[13] *Z. phys. Chem.*, **76**, 179 (1911).
[14] *J. Chem. Soc.*, **119**, 1369, 1374, 1669 (1921).

His results further indicated that sodium chloride is slightly adsorbed by the curd fibres to an extent of not greater than 1 per cent of their weight when the sodium chloride was saturated.

More recently Miss Laing has investigated quite a large number of substances with a view to finding a reliable and convenient reference substance, for even sodium sulfate is appreciably adsorbed unless there is a high concentration of another salt present in the liquid. Of all substances investigated, glycerine alone is not appreciably taken up by the fibres, and Miss Laing's results with this substance gave values in accordance with the previous work.

Wintgen [15] claims that the reciprocal of the density, the specific volume, is practically a linear function of the percentage concentration by weight. This was tested with arsenic sulfide, antimony sulfide, silicic acid, molybdic acid, ferric hydroxide, tannin, starch, gelatin, albumin, serum globulin, etc. If extrapolation is made to hundred per cent colloid, the value for the specific volume comes out too low and consequently the value for the density too high. This is probably due to the existence of condensed water around each particle.[16]

An anhydrous substance in suspension such as ferric hydroxide, arsenic sulfide, gold, etc., appears to have no effect on the surface tension of the medium; but some of the gelatinous colloids have a marked effect. Soap lowers the surface tension a great deal and gelatin also causes a marked decrease of the surface tension of water.

It seems probable therefore that hydrophile colloids affect appreciably the surface tension of the medium, whilst hydrophobe colloids do not alter the surface tension.

In the case of water-soluble colloids, the viscosity may increase enormously with the concentration, a 1 per cent solution of agar forming a solid jelly. The metal and the sulfide sols have only a very slight effect on the viscosity of water. This varies with the nature and subdivision of the solid particles. According to Hatschek 20 to 30 per cent of precipitated calcium carbonate or barium sulfate does not increase the viscosity very much, on the other hand Bingham [17] found zero fluidity or infinite viscosity with 4 per cent china clay or 5.5 per cent of graphite. Oden [18] found an approximately 50 per cent greater viscosity with sulfur sols, in which the particles had a diameter of about 10 mμ than with sols in which the sulfur particles had a diameter of 100 mμ. Hatschek [19] attributes this to the existence of an adsorption film of liquid round the sulfur particles. Hatschek has deduced a formula for the viscosity of two liquid phases in which the volume of the disperse phase is the larger.

$$\eta_1 = \eta \frac{\sqrt[3]{A}}{\sqrt[3]{A-1}}$$

where $A = \dfrac{1}{f}$ and is the ratio of the total volume to the volume of the disperse phase; from this formula he deduces that one volume of casein binds more than eleven volumes of water and that in a 0.5 per cent rubber sol the rubber binds 75 to 100 volumes of liquid. Applying the same calculation to Bingham's experiment on zero fluidity with graphite and water it is found

[15] *Kolloid chem. Beihefte,* **7**, 251 (1915).
[16] Compare *Kolloid Z.,* **20**, 239 (1917).
[17] *J. Am. Chem. Soc.,* **46**, 278 (1911).
  J. Franklin Inst., **181**, 845 (1916).
[18] *Z. phys. Chem.,* **80**, 709 (1912).
[19] *Kolloid Z.,* **11**, 280 (1912).

out that each volume of graphite must adsorb about nine volumes of water.[20] These results are of a qualitative nature and want confirmation by the measurement of vapor pressure for mixtures of the substances with water. If the particles form into chains, the viscosity will be increased very much. If the particles form larger spherical particles which are homogeneous there will be a decrease of viscosity because of the decrease in the surface and consequently in the amount of the bound water.

The important point is, that increase in viscosity goes hand in hand with an increase in agglomeration,* which means that increasing agglomeration involves decrease in the amount of available free water. The phenomenon seems general because Freundlich [21] has made use of the increase in viscosity as a means of studying the rate of agglomeration of sols of aluminium hydroxide. With colloidal solutions of gelatin and other substances of the same type, the viscosity changes with the time, showing there is a gradual change in structure. This is further confirmed by the fact that the viscosity of such solutions changes when they are shaken violently.

We shall now discuss the question of viscosity of sols in presence of electrolytes. We know that if we add potassium iodide to a gelatin jelly, it melts into a liquid. We can explain this phenomenon in the following way: The particles of jelly have a chemical attraction for the iodide ions, and they adsorb these ions with the formation of a negatively charged sol. Now these charged particles of a micellar nature tend to shrink and squeeze out the adsorbed water; consequently the jelly containing potassium iodide passes into the liquid condition and forms a negatively charged colloid. If a bivalent salt like $CaCl_2$, or $SrCl_2$ is added to this liquid, the whole thing sets into a jelly again, because the negatively charged particles of the jelly adsorb the bivalent Ca ions and become neutral, and as soon as they become neutral they adsorb again the water molecules which they have squeezed out, because of their previous charge. Consequently, we are of the opinion that sols carrying a charge are less hydrated than the freshly coagulated mass of the same substance.

In this connection it is interesting to observe that it has been shown that ions occupy less space than molecules.[22] Moreover, it has been observed that elements with low ionisation potentials have large atomic volumes, e.g., metallic cæsium has an ionisation potential smaller and atomic volume greater than that of lithium.

Lillie [23] has shown that cilia of the larva of a ringworm *Arenico Arenicola,* as before mentioned, is liquefied by a solution containing sodium ion, the addition of a small amount of a divalent cation, stops the process. We can explain the above facts and also the biological salt antagonism in the following way: Cells in the larva of the ringworm (or any other animal matter) are of an albuminous nature, and like albumin they are either weakly negative or weakly positive or neutral. We can assume that the cells are either neutral or carry a very small negative charge. In presence of NaCl, the cells would adsorb Cl ion due to chemical affinity and would become negatively charged, and would probably squeeze out the adsorbed water, and consequently the larva would pass into the liquid state. Now the addition of a little bivalent calcium ion

[20] Compare Bancroft, "Applied Colloid Chemistry." page 192
 * The contrary is the case when we are on the other side of the zone of maximum colloidality. See Vol. I, Chapter I, of this series. *J. A.*
[21] *Trans. Faraday Soc.,* **91** 66 (1913).
[22] *Z. Elektrochem.,* **19**, 748 (1913).
[23] *Am. J physiol.,* **10**, 433 (1904).

would neutralize the negative charge and the particles of larva will readsorb the water it has previously squeezed out because of its charge and micellar nature, and thus reestablish the original condition of the larva.

There is chemical analogy of this phenomenon of the change of mobility due to the adsorption of an ion. When NaOH is gradually added to a fairly concentrated solution of $CuSO_4$, $Cu(OH)_2$ which is formed must be positively charged due to the adsorption of positive ions and the mixture is very mobile. If the addition of alkali is continued, a certain stage appears when the mobility of the system decreases considerably and a viscous bulky precipitate of $Cu(OH)_2$ is obtained. At this stage the whole of the copper is precipitated and the hydroxide is practically neutral. If more alkali is added, the mixture becomes again mobile and $Cu(OH)_2$ becomes negatively charged due to the adsorption of hydroxide ions and the precipitate becomes less bulky. It is apparent therefore that the charged hydroxide of copper squeezes out some water of hydration which it can take up again on charge neutralisation. This phenomenon seems to be general in the precipitation of many substances."

From the above quotation it will be seen that we have made the following assumptions :

(1) Other things being identical, the uncharged substance is more hydrated than the sol.

(2) The greater the hydration of a substance the greater is its viscosity, and

(3) When a sol adsorbs an ion carrying the same charge as the sol, because of chemical affinity, the charge on the sol is increased and consequently according to our view the viscosity of the sol should decrease.

We shall show in the subsequent pages that these assumptions are corroborated by the experimental results of various workers on the viscosity of colloids in presence of electrolytes.

In a series of papers [24] published from these laboratories, we have proved qualitatively and quantitatively that many sols like As_2S_3, Sb_2S_3, mastic, Prussian blue, etc., are capable of adsorbing ions carrying the same charge as the sol, and are stabilised by this adsorption of ions carrying the same charge as the sol. We are trying to prove experimentally that when small quantities of electrolytes insufficient to coagulate the sols are added to them, these sols adsorb ions carrying the same charge as the sol, and the viscosity is slightly decreased. On the other hand, sols like $Fe(OH)_3$, $Cr(OH)_3$, etc., which adsorb ions of the same charge as the sol to a very slight extent, should show much less decrease of viscosity on the addition of small quantities of electrolytes than when small quantities of electrolytes insufficient for coagulation are added to sols As_2S_3, Sb_2S_3, etc. Moreover we have proved that when ferric hydroxide is coagulated by ferric chloride, aluminium nitrate, etc., the sol adsorbs ferric ions, Al-ions, etc. Consequently we venture to suggest that when small quantities of ferric chloride or aluminium nitrate is added to a sol of ferric hydroxide, there will be more marked decrease of viscosity than when KCl, $KBrO_3$, etc., are added to the sol. We are of the opinion that those sols which behave abnormally towards dilution, towards a mixture of electrolytes of different valencies, and show positive acclimatization, should

[24] *J. Phys. Chem.*, **28**, 313, 457 (1924); **29**, 435, 659 (1925).
Kolloid Z., **34**, 262 (1924); **35**, 144 (1924).

also show more appreciable decrease of viscosity when small quantities of the precipitating electrolyte is added to them than those sols which are normal towards dilution, towards a mixture of electrolytes and show the phenomenon of negative acclimatization. All these phenomena mainly depend, as we have already proved, on the amount of adsorption of ions carrying the same charge as the sol. It must be emphasized that the change in viscosity of a sol on the addition of an electrolyte, will certainly depend on the ratio of the adsorption of the positive and the negative ions. If the sol adsorbs more of the ion carrying the opposite charge than of the ion carrying the same charge, the charge on the sol is decreased and more hydration will take place and the viscosity would be increased. On the other hand, when a sol is capable of adsorbing more of the ion carrying the same charge than of the ion carrying the opposite charge, the viscosity should decrease. We have made the tacit assumption that the degree of dispersion remains the same in all cases.

These conclusions are corroborated by the following experimental results.

Albanese [25] has observed that the viscosity of an aqueous solution of gum arabic is lowered to the extent of 30 per cent by the addition of small quantities of electrolytes but not by other substances.

It seems that gum arabic is capable of adsorbing ions carrying the same charge as the sol. Woudstra [26] has shown that the viscosity of ferric hydroxide or chromic hydroxide sol slightly decreases on the addition of small quantities of electrolytes; when greater quantities of electrolytes are added the viscosity goes on increasing. Moreover he has shown that the viscosity of colloidal silver is lowered appreciably by the addition of electrolytes. From an experimental work which is in progress in these laboratories we find that silver sol can adsorb ions carrying the same charge as the sol and is abnormal towards dilution and to a mixture of electrolytes, and would show the phenomenon of positive acclimatization; consequently our conclusion that this sol should show appreciable decrease of viscosity on the addition of small quantities of electrolytes is corroborated by the experimental work of Woudstra.

Recently we have observed that there is first a decrease in viscosity when an electrolyte like KCl is added to an undialysed vanadium pentaoxide sol. When more of the electrolyte is added the viscosity continually increases.

Farrow [26a] has shown that the viscosity of sodium palmitate solution decreases on the addition of small quantities of NaOH, NaCl, KCl; if the amount of the electrolyte is increased the viscosity, after passing through a minimum, goes on increasing. These results are very likely due to the adsorption of ions carrying the same charge as the sol of sodium palmitate. If we assume that the colloidal particles existing in sodium palmitate are negatively charged, we can expect that these negatively charged particles will adsorb OH or Cl ions because of the chemical affinity of the particles of palmitate for OH or Cl ions, when small quantities of alkali or chloride is added to the solution of the palmitate; and hence the charge is increased and the viscosity decreases. Now, if more of the electrolyte is added, the oppositely charged ion is adsorbed in greater quantities and the charge on the colloidal matter is neutralised and hence the viscosity and hydration go on increasing. In this connection it will be interesting to observe that Salmon [27] has shown that NaCl is appreciably adsorbed by soap. Also it is well known that free alkalies are adsorbed by

[25] *Arch. Exp. Path.*, Pharm. Suppl., 16 (1908).
[26] *Z. phys. Chem.*, 63, 619 (1908).
[26a] *J. Chem. Soc.*, 101, 347 (1912).
[27] *J. Chem. Soc.*, 119, 1369, 1374, 1669 (1921).

soaps. Egnar [28] has shown that cations flocculate suspensions of china clay and infusorial earth and increase the viscosity, but anions act in the opposite way. The magnitude of the flocculating power generally follow the valency rule, but hydroxide ions often act irregularly in this case, as well as in the cases of many other sols. The properties of these suspensions is very similar to those of colloids, and apparently these suspensions are negatively charged.

Some very interesting results have been obtained by Fernau and Pauli [29] on these lines. They have found that on the addition of a quantity of an electrolyte which is not sufficient to produce coagulation, in the case of the sol of cerium hydroxide there is an immediate drop in the viscosity of the sol. We are of the opinion that this is due to an increase in the charge of the sol by the adsorption of the ion carrying the same charge as the sol.

The above authors have also proved that the sol of ceric hydroxide becomes less viscous on "ageing." In the foregoing pages we have emphasized that ageing is essentially connected with dehydration specially of hydrophobe colloids, hence an aged sol of cerium hydroxide loses its water and becomes less viscous. We have already suggested that this phenomenon of decrease of viscosity and of dehydration on ageing are of common occurrence.

Büchner [30] has shown with sols of molybdenum-blue and $Fe(OH)_3$ that the density of the suspended particles is much smaller than that of the substances in the free condition. This is interpreted in favor of the view that the colloidal particles contain water; the proportion of water which thus becomes associated with the colloid is greater for $Fe(OH)_3$ than for molybdenum-blue.

Odén [31] concluded from theoretical considerations that the viscosity of suspensoids should be independent of the degree of dispersion of the colloid. He however experimentally found an approximately 50 per cent greater viscosity with sulfur sols in which the particles had a diameter of 10 mμ than with sols of diameter of 100 mμ. This is certainly due to the greater amount of water adsorbed by the increased surface of the smaller sulfur particles.

Hatschek [32] has deduced that the thickness of water films round the sulfur particles is about 0.87 mμ, which corresponds to an increase in volume of about 62 per cent for particles having a diameter of 10 mμ. Hence the greater hydration of the particles is associated with the increase in viscosity. Alexander [33] has shown with Karaya gum that the viscosity of emulsoids or hydrophile colloids increases as the dispersed phase becomes finer.* This is also due to the greater amount of water adsorbed by the fine particles of the colloid.

Moreover Miss Chick [34] has shown that 2.1, 3.8, and 5.8 cc. of water are adsorbed per gram of protein with serum albumin, globulin, ψ globulin and euglobulin respectively at 25°. Hence by the withdrawal of water in any salting out process the euglobulin requiring most water is the first, and serum albumin is the last to be precipitated.

In the case of water-soluble colloids the viscosity may increase enormously with the concentration of the colloid; a one per cent solution of agar forms a solid jelly. The metal and the sulfide sols have only a very slight effect on

[28] *Medd. K. Vetnsk. Nobel. Inst.*, **4**, 41 (1920).
[29] *Kolloid Z.*, **20**, 20 (1917).
[30] *Proc. Akad. Wetensch. Amsterdam*, 18, 170 (1915).
[31] *Z. phys. Chem.*, **80**, 709 (1912).
[32] *Kolloid Z.*, **7**, 301 (1910).
[33] *J. Am. Chem. Soc.*, **43**, 434 (1921).
* This increase reaches a maximum and then falls as molecular dispersion is approached. See Chapter 1, Vol. I, this series. *J. A.*
[34] *Biochem. J.*, **8**, 261 (1914).

the viscosity of water. The fact that the viscosity of many colloidal solutions changes more rapidly than the concentration is not in agreement with Einstein's formula [35] and Hatschek [16] has expressed the views that this is due to the formation of an envelope of the medium round each particle of the dispersed phase. Both Arrhenius [37] and Smoluchowski [38] are not in favor of this view of Hatschek. Smoluchowski has also remarked that on various occasions it has been suggested that the increase in viscosity on coagulation of a colloid is directly opposed to Einstein's formula. This increase Smoluchowski ascribes to the formation of non-spherical aggregates. The same author finds greater difficulty in explaining such cases where the addition of a small quantity of an electrolyte results in the diminution of the viscosity of the sol, and he throws out the suggestion that the decrease of viscosity is due to the reduction of the volume of dispersed phase. We venture to suggest that the origin of these difficulties of Smoluchowski lies in the assumption that the viscosity increases with the increase in the charge of the colloids. On the other hand, our view that the uncharged particles are more hydrated and more viscous than the charged particles under identical conditions, seems to be more consistent with the experimental observations and immediately solves the difficulties of Smoluchowski.

Moreover Getman [39] has shown that in every case the viscosity-concentration curves of some solutions of potassium salts having lower viscosities than that of the solvent, pass through a minimum and it is suggested that this abnormal behavior was due to the combined action of ions and the undissociated molecules. The potassium ions appear to lower the viscosity of the solvent while the anions and undissociated molecules tend to increase it.

In this connection the following remarks of Bancroft [40] will be of interest. "If the suspended particles aggregate into chains, the viscosity will be increased very much. If the particles form larger spherical particles which are homogeneous, there will be a decrease in the viscosity, because of the decrease in the surface and consequently in the amount of the bound water. If however the particles simply agglomerate loosely into spherical masses, the viscosity will increase, because the water in the voids inside the spherical agglomerates no longer counts as free water. We shall therefore expect to get an increase of viscosity as a result of agglomeration when the effect of agglomeration is not to increase the size of homogeneous drops." The important point is that the increase in viscosity does go hand in hand with an increase in agglomeration, which must mean that increase in agglomeration involves decrease in the amount of available free water.

Although there has been an immense amount of work done on the viscosity changes of the substances like gelatin, blood, etc., no very definite conclusions have yet been arrived at. Bancroft has remarked: [41] "With colloidal solutions of gelatin and other substances of the same type, the viscosity changes with time, showing that there is a gradual change in structure. This is further confirmed by the fact that the viscosity of such solutions changes when they are shaken violently."

Where a film formation is possible, the surface viscosity will be quite dif-

[35] *Drudes Annalen.* **19**, 289 (1906).
[36] *Loc. cit.*
[37] *Medd. Nobel Inst.*, No. 13, **16**, 1 (1916).
[38] *Kolloid Z.*, **18**, 190 (1916).
[39] *J. Am. Chem. Soc.*, **30**, 728 (1908).
[40] "Applied Colloid Chemistry," 1921, p. 192
[41] "Applied Colloid Chemistry," 1921, p 195

ferent from that in the mass of the liquid. This is very striking in the case of colloidal solutions of saponin, peptone, etc."

Gokun [42] has reported that the viscosity of 0.28 per cent gelatin solution increases with time by 1½ in 115 hours. The viscosity depends on the mechanical treatment to which the solution has been subjected. This points to the existence of structure in the solution.

Gunzburg [43] has shown that viscosity of muscle juice from a frog decreases on the addition of KCl whilst it increases in presence of uranium nitrate. It appears that the juice is negatively changed and becomes more stable and less viscous by the adsorption of Cl ions from KCl. Whilst the bivalent uranium ions reduce the charge and increase the viscosity of the juice. In the presence of a mixture of the two salts the viscosity depends on the proportions of the two salts present.

Moreover Rothlin [44] has divided hydrophile colloids into two groups, one of which follows Poiseuille's law whilst other does not. These deviations are to be ascribed to the formation of larger aggregates in the sol through gelation; these aggregates, according to the condition of flow, can be broken down to different extents and so give rise to the observed irregularities. Bungenberg de Jong [45] concludes that for viscometric measurements to have any value, the system under examination must not only follow Poiseuille's law but the dispersed phase must retain its stability. Consequently with some hydrophile colloids the variation in the distribution in the shears in the liquid play an important part. Recently Freundlich and Schalek have observed that certain inorganic colloids such as vanadium pentoxide, old $Fe(OH)_3$, etc., do not follow Poiseuille's law.·

It is not easy to explain all the viscosity experiments in presence of electrolytes with substances like gelatin, albumin, blood casein, etc., from a single point of view. Two main workers in this field, Pauli and Loeb, do not agree with each other's conclusions as will be evident from the following quotations from Loeb's book: "The idea that the viscosity of protein solution depends primarily upon the protein ion was accepted by Pauli who made the additional hypothesis that each protein ion is hydrated; i.e., that each individual protein ion is surrounded by a considerable shell of water. Pauli worked with blood albumin which had been freed from salts by a dialysis continued for several weeks. When he added acid to water soluble albumin the viscosity increased first from 1.0623 for the pure albumin solution to 1.2937 when the concentration of HCl added to the albumin solution was 0.17 N when the HCl concentration was increased to 0.05 N the viscosity was only 1.1667. The following figures give the data according to Pauli:

Concentration of HCl	0.0N	.005N	.01N	.012N	.017N	.02N	.03N	.04N	.05N
Viscosity	1.0623	1.2555	1.233	1.274	1.2937	1.277	1.224	1.1822	1.1667

Pauli assumed that the protein ions are surrounded by a jacket of water, whilst the non-ionized molecules of protein he assumed not to be hydrated. Addition of a little HCl to isoelectric albumin would cause the transformation of non-ionized albumin into albumin chloride which is highly ionized and hence assumed to be highly hydrated; the more acid is added the more

[42] *Kolloid Z.*, **3**, 84 (1908).
[43] *Arch. Teirland Physiol.*, **4**, 233 (1920).
[44] *Biochem. Z.*, **98**, 34 (1919).
[45] *Rec. trav. Chim.*, **42**, 1 (1923).

albumin chloride and more hydrate albumin ions should be formed. Hence the viscosity should at first increase with the quantity of acid added, until a point is reached where the addition of more acid represses the degree of electrolytic dissociation of the albumin chloride on account of the high concentration of the Cl ion common to both protein chloride and HCl." On page 115 Loeb remarks:

We have shown that the curves for osmotic pressure, swelling and viscosity reach a maximum at a pH varying between 3.5 and 2.8 and that they then drop. Pauli assumes that the drop is due to a repression of the degree of electrolytic dissociation of the gelatin chloride (or any protein-acid salt) through the addition of more acid on account of the common anions. It should, however, be mentioned that Pauli and Mauabe and Matula state that the maximum of the curves occurs not at pH between 3.5 or pH 2.8, but at pH 2.1 or 2.0.

The hydration hypothesis can be put to a direct test by determining the specific conductivity of solutions of protein salts e.g. gelatin chloride, albumin chloride, etc. Since according to the hydration hypothesis only the protein ion undergoes hydration, the variation in the osmotic pressure, swelling and viscosity should be accompanied by a corresponding variation in the concentration of the protein ions in solution. If therefore the specific conductivity of gelatin chloride is measured at varying pH but equal concentrations of originally isoelectric gelatin, the curves representing the values found for conductivity of the protein should run parallel with the curves for the osmotic pressure, swelling, and viscosity; moreover the curve for the conductivity of gelatin sulphate should be only about half as high as the curve for the specific conductivity of gelatin chloride; while the curve for the specific conductivity of gelatin oxalate should be almost but not quite as high as that of gelatin chloride. The experiments show that this is not the case.

Figure 39 on page 118 shows that the same disagreement exists between the conductivity curve and the osmotic pressure curve for solutions of the chloride of crystalline egg albumin. These curves then do not support the hydration hypothesis.

Pauli's hydration theory rests as stated above on an assumption made by Kohlrausch that the difference in the mobility of ions is due to molecules of water being dragged along with the migrating ion. Lorenz, Börn and others have come to the conclusion that while Kohlrausch's idea is probably correct for monatomic ions, it cannot be correct for large polyatomic ions. This would exclude the assumption of a high degree of hydration of protein ions.

It is well known that small quantities of salts of heavy metals like Ag, Cu, Zn, Co, Pb, etc., can readily coagulate albumin. This is because the negatively charged albumin has a marked chemical affinity and adsorbs preferentially these heavy positive ions. In this connection it will be interesting to note that the negatively charged MnO_2 sol has a great affinity for heavy positive ions like Ag, Cu, Zn, etc., and is readily coagulated by these ions.

The fact that the viscosity of a 5 per cent solution of isoelectric gelatin increases rapidly at the temperature of 20° or below cannot possibly be explained on the basis of the hydration theory of Pauli since isoelectric gelatin is not ionized. This fact can be satisfactorily explained because we have assumed from our point of view that the uncharged substance is more hydrated and more viscous than the charged particles under otherwise identical conditions. Consequently isoelectric gelatin which is very feebly charged becomes more and more hydrated and viscous by the adsorption of water.

It is well known that colloids like ferric hydroxide, arsenious sulfide, gold, etc., do not appreciably affect the surface tension of water, but some of the gelatinous colloids have a marked effect. Addition of soap decreases the surface tension of water to a great extent. The addition of small quantities of gelatin to water markedly decreases the surface tension of water. We must emphasize that the changes in a colloidal system need not always consist in the diminution of dispersion. Occasionally we find that the particles be-

come smaller with lapse of time, and th⬛⬛s hitherto been observed only in the case of hydrophile colloids, e.g., glyco⬛⬛, benzopurpurin and hæmoglobin, lecithin, etc.

If we assume that the particles of a sol of gelatin or albumin, etc., have a natural tendency to disintegrate, we can satisfactorily explain the viscosity measurements of these substances. In the case of isoelectric gelatin, according to this point of view, the suspended particles are becoming smaller and smaller because of their natural tendency for disintegration, and consequently these smaller particles will adsorb more water and the viscosity of the system will increase.

When MCl is added to isoelectric gelatin the gelatin becomes positively charged by the adsorption of hydrogen ions, and the particles of gelatin containing more charge than the particles of isoelectric gelatin have a greater tendency for disintegration than the particles of isoelectric gelatin itself. Hence in presence of HCl the degree of dispersion of albumin will increase and along with it the hydration and viscosity will also increase. Now in presence of larger quantities of HCl the adsorption of negatively charged Cl ion by the positively charged gelatin will become appreciable and consequently the charge on the gelatin will not rise proportionately with the increase in concentration of HCl and after a time the charge will have a tendency to decrease by the adsorption of Cl ions. As soon as the charge on the gelatin is decreased, the degree of dispersion will also decrease and along with it the hydration and viscosity will also decrease. Hence we can explain the viscosity curves for gelatin, albumin, etc., with definite maxima on the addition of acids or alkalis. From the disintegration point of view the increase in viscosity on the addition of alkalis to albumin, gelatin, etc., can also be readily explained in the following way :

When small quantities of alkali are added to gelatin or albumin OH ions are preferentially adsorbed and the charge on the albuminous matter is increased, consequently the disintegration tendency and the degree of dispersion of the particles of albuminous matter are increased. As soon as the degree of dispersion is increased more of hydration of the substance takes place and the viscosity increases. Now if more and more of alkali is added the adsorption of OH ions will increase, but after a time a limit will be reached because at this stage the influence of the positive ion will be felt due to the adsorption of Na, Ca ions, etc., from the alkali. Along with the decrease in charge the degree of dispersion will decrease and subsequently the amount of hydration and viscosity will decrease.

Loeb has shown that the viscosity of gelatin, albumin, etc., increases much less in presence of H_2SO_4 than HCl of the same concentration. This is due to the fact that the increase in the charge due to the adsorption of H^+ ions and consequent increase in disintegration of albuminous matter will be less pronounced with H_2SO_4 than with HCl because of the more pronounced effect of the oppositely charged bivalent SO_4 ions. Similarly from the experimental results of Loeb we find that increase in viscosity of casein, gelatin, etc., is more pronounced when KOH, NaOH, etc., are added than with $Ca(OH)_2$, $Ba(OH)_2$, etc. This is also due to the fact that the increase in charge due to the adsorption of OH ions and consequent increase in the degree of dispersion will be less pronounced with $Ca(OH)_2$, $Ba(OH)_2$, etc., than with NaOH, KOH, etc., because of the more pronounced effect of the oppositely charged bivalent ions Ca, Ba, etc., in decreasing the charge.

From this point of view increased swelling in presence of acids or alkalis is due to the fact that in presence of them the degree of dispersion is increased and along with it the amount of hydration will also increase. It must be emphasized that the explanation of the increase in viscosity from this point of view of increased dispersion, is only applicable to those hydrophile colloids which show a decrease in surface tension of the solvent when these substances are added to the solvent.

The Surface Tension of Colloids With Special Reference to Protein Colloids

By Dr. Fil. Bottazzi,*

Naples

Introduction

For the most part the information given in the treatises on physical and colloidal chemistry concerning the influence exerted on the surface tension of water by hydrophilic colloids in general and protein substances in particular does not go beyond the early researches of Quincke, Traube and other experimenters. While Bottazzi and his students have carried on the major portion of investigations on the surface tension of protein solutions and suspensions, the results of these researches have not found their way into the above-mentioned treatises.

It is necessary first of all to establish the fact that protein substances can lower the surface tension of water.[1] Traube[2] denies this in one of his publications ("Die Osmotische Kraft," 1908) where he states (page 432): "While emulsions . . . and albumin hardly lower the surface tension of water at all, peptones and albumoses lower it very powerfully. . . ."

Rona and Michaelis,[3] basing their researches on the statements of Traube, have ignored the work of Bottazzi published not only in Italian[4] but also in German.[5] Brailsford Robertson[6] pays scant attention to the surface tension of protein solutions, and Bechhold[7] merely says that "proteins may be strongly adsorbed, or, on the other hand, exert a powerful adsorption," without specifying the conditions of adsorbent action or of adsorption. Moreover, he adds: "Proteins are frequently used as adsorbent both in a solid and in a denatured condition. . . . Adsorption by protein in solution is more important than adsorption by solid proteins."

Höber[8] echoes this uncertainty in the following words: "The surface tension of solutions of hydrophile colloids is certainly very variable, one reason being, as we shall see, because, under varying conditions, the condition of hydrophile colloids may approach more or less closely that of suspension colloids. Therefore we find reports that, e.g. proteins do not affect the surface tension of water at all, as well as reports of actual and relatively large surface activity. Prolonged dialysis or purification by precipitation and re-solution may increase the suspensoid character with corresponding reduction of the

* Translated by Prof Lawrence T Fairhall, A M Ph D, Harvard
[1] Here other solvents or dispersion media are not taken into account.
[2] J. Traube, Pfluger's Archiv., 123, 419 (1908).
[3] P. Rona u. L. Michaelis, Biochem. Z., 41, 165 (1912).
[4] Fil. Bottazzi, Arch. Fisiol., 7, 579 (1909).
[5] Fil. Bottazzi, C. Neuberg's "Der Harn, etc.," p. 1715, Berlin, 1911. Kolloid-Chem. Beihefte, 3, p. 165, 173 segg., 1912.
[6] T. Brailsford-Robertson, "Die physikalische Chemie der Proteine," Dresden, 1912.
[7] H. Bechhold, "Colloids in biology and medicine." Translated by J. G. M. Bullowa. New York, 1919, p. 143.
[8] R. Hober, "Physikalische Chemie der Zelle und der Gewebe," V Aufl., p. 177, Leipzig, 1922.

surface activity. The same a̶▉▉▉ to glycogen, starch, lecithin, soaps, dyes, etc."

Freundlich [9] alone admits (with reserve) the possibility that protein substances lower the surface tension. In fact he writes: ". . . thus proteins,[10] gum, etc., lower the surface tension of water rather strongly, soaps,[11] saponin, etc., very strongly. Certainly prior work along this line must be closely scrutinized. For it is well known that minute amounts of capillary-active substances suffice to lower the surface tension sharply, and the hydrophile sols in question are seldom so carefully purified that it can be safely said that the surface tension lowering observed is consequent on the action of the micellae themselves, and not of incidental impurities. For instance it may be stated that carefully purified casein lowers the surface tension of water but slightly, whereas commercial casein lowers it markedly." [13]

What Freundlich says of casein is quite true; in fact, Merck's casein, notoriously impure, lowers the surface tension of water markedly (from 55 drops to 74 drops with Traube's stalagmometer). By the addition of NaOH to a 2 per cent solution, the suspension is transformed into a solution without greatly increasing the stalagmometric reading (74 to 75 drops). But Kahlbaum's casein, prepared by Hammarsten's method, washed with ether, etc., gave the following results: [14]

Distilled water (28° C.) .... 55 drops
2 per cent submicroscopic suspension of Kahlbaum's casein. .... 60 drops
The same suspension after the addition of 6 drops of NaOH solution 66 drops
5 per cent solution of the same casein........... .... 75 drops
Very concentrated and viscous solution of the same casein.......... 80 drops

This decrease in surface tension with increasing concentration would not have been so great if the substance lowering the surface tension were an impurity of the casein, as for example some fatty acid.

But no doubt can arise with measurements made upon suspensions and solutions of serumglobulin which has been thoroughly dialyzed. This question is of such importance that it would seem of value to review briefly the principal results obtained by Bottazzi from 1908 to 1912.

SURFACE TENSION OF PROTEIN SUSPENSIONS AND SOLUTIONS

Bottazzi observed variations in determining the viscosity [15] and surface tension [16] of the blood of *Maja squinado* dialyzed for several days (until it became opalescent and non-coagulable by heat) on addition of increasing amounts of NaOH. These results [17] are summarized in the following table and in Figure 1.

[9] H. Freundlich, "Kapillarchemie," 2nd ed., p. 733, Leipzig, 1922.
[10] Quincke, *loc. cit.*: Bottazzi, *Rend. accad. Lincei* (5), 21 (2° sem.), 221 (1912); Bottazzi and d'Agostino, *ibid* (5), 21 (2° sem.), 561 (1912).
[11] Rayleigh, *Proc. Roy. Soc. (London)*, 47, 281 (1890), Bottazzi and Victorow, *Rend. accad. Lincei* (5), 19 (1° sem.), 659 (1910); Bottazzi, *ibid* (5), 21 (2° sem.), 365 (1912).
[12] Rayleigh, *loc. cit.*
[13] Berczeller, *Biochem. Z.*, 53, 232 (1913).
[14] Fil. Bottazzi, *Rend. accad. Lincei* (5), 21 (2° sem.), 221 (1912).
[15] With an Ostwald viscosimeter having a distilled water value of '23, 5° C. = 1'25".
[16] The method of G. Fano and M. Mayer (*Arch. Fisiol.*, 4, 165 (1907)). The value of *h* with distilled water is little different from that found for dialyzed opalescent *Maja* blood (*h* for H₂O = 54.2). As one knows, the values of *h* vary with those of the surface tension of the liquid examined; for this reason with a given liquid of constant colloidal concentration, specific gravity, and temperature, and a given capillary, the values obtained for *h* are quite comparable.
[17] Fil. Bottazzi, *Arch. Fisiol.*, 7, 579 (1909).

TABLE 1

		Concentration of NaOH in Liquid	Viscosity (t) at 23.5° C.	Surface Tension (h) at 24° C.
Opalescence Decreasing		N	*Seconds*	*mm.*
		0	166	51.5
		0.0001	169	....
		0.0002	181	....
		0.001	....	42.5
Limpid Liquid		0.005	214	....
		0.01	242	36.5
		0.05	206	38.3
		0.075	202	....

From Table 1 and from Figure 1 it follows that the viscosity and surface tension of the blood of *Maja squinado* (that is, of a suspended solution of protein freed from salts and approaching the isoelectric point) upon the addition of NaOH, are affected differently; with increasing concentration of NaOH the viscosity rapidly increases while the surface tension rapidly diminishes. The opalescence gradually disappears, and at a concentration of 0.005 N
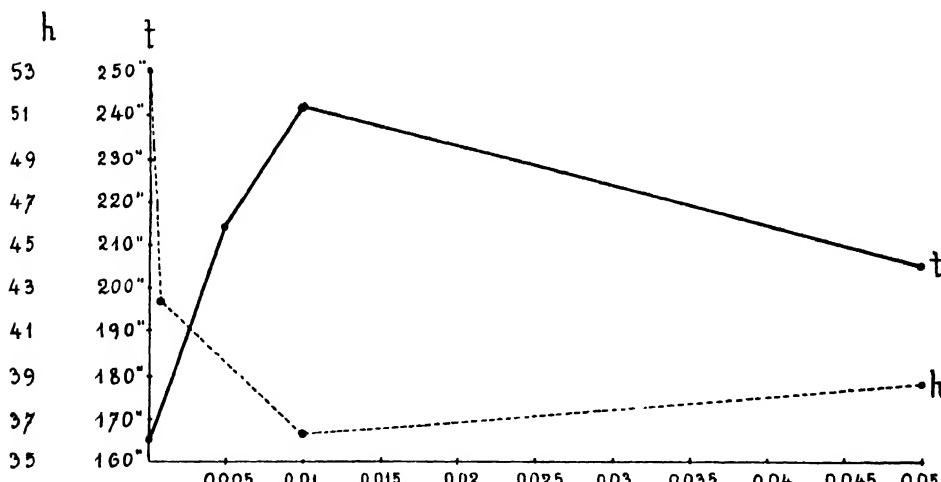
Fig. 1.

NaOH the liquid becomes limpid. The surface tension of dialyzed blood differs little from that of pure water. This is explained by the fact that in the dialyzed blood the colloids exist in a state of submicroscopic suspension. In fact we know that suspensoids have a surface tension little different from that of the dispersion medium.[18] The opalescent, milk-white appearance of the blood indicates an extremely fine suspension of colloids. But NaOH acts on the colloidal suspension, transforming the opalescent liquid into a limpid

[18] "da die suspendierten Teile die Oberflachenspannung nicht erniedrigen." H. Freundlich, "Kapillarchemie," pp. 310 and 313, Leipzig, 1909.

colloidal solution. As a consequence the surface tension is lowered. In fact we know that "the surface tension of emulsion colloids . . . differs markedly from that of the pure dispersion medium, and that of water is often materially lowered by the presence of colloidally dissolved substances."[19]

Determinations of surface tension of dispersoids compared with that of water, therefore, enable us to distinguish a suspensoid from an emulsoid, and indicate the point at which a colloid in a given liquid passes from a state of suspension to that of solution or vice versa.

Experiments with suspensions of serum globulin of mammalian blood confirm these results.[20]

Experiment: solution-suspensions of purest serum globulin in a mixture of 1 volume of normal NaCl solution to 2 volumes of normal KCl solution. The liquid is turbid, milky, because it contains much suspended globulin.

Viscosity: H₂O ....(t)...at 25° C. = 2 min. 31⅔ sec.
      Solution-suspension of globulin ... .at 25° C. = 2 min. 54⅔ sec.
Surface tension: H₂O. ....(h)... at 25° C. = 54.2 mm.
      Solution-suspension of globulin...... .....at 25° C. = 35.2 mm.

TABLE 2.

Cc. of Globulin Suspension	1 cc of	Total NaOH Concn.	Viscosity at 25° C. (t) Min Sec.		Surface Tension at 25° C. (h) Mm.
4	H₂O	0	2	54⅔	35.2
4			2	42	37.0
4	NaOH n/100	n/500	2	46⅓	36.1
4	NaOH n/50	n/250	2	42⅔	36.1
4	NaOH n/20	n/100	2	40	37.0
4	NaOH n/10	n/50	2	40	37.2

It is apparent that the addition of NaOH at first increases the viscosity and lowers the surface tension; as the NaOH concentration increases, however, the viscosity is lowered and the surface tension increased. The effect of the NaOH is not marked in this case because of the salt concentration (with respect to effect of NaOH alone, see above).

In researches made by Bottazzi with the assistance of Buglia and Jappelli,[21] it was observed that the surface tension of mammalian blood serum diminishes during dialysis at first rapidly, then more slowly. This reduction is most noticeable during the first 5 hours of dialysis during which the serum becomes turbid and then commences to precipitate. The decrease of surface tension is apparent whether one removes the flocculates or not. The surface tension of normal blood serum is lower than that of pure water, and it is reasonable to assume that the decrease in surface tension of water is mostly caused by the proteins. Now the precipitation of one part of the proteins, and the consequent dilution of the serum with water should increase the surface tension. Instead this diminishes little by little and remains lower than that of water after the separation of the globulin precipitate.

This accords with Buglia's observations[22] that simple dilution of blood

[19] H. Freundlich, loc. cit., p. 393.
[20] Fil. Bottazzi, Arch. Fisiol., 7, 601-602 (1909).
[21] Fil. Bottazzi, G. Buglia and A. Jappelli, Rend. accad. Lincei (5), 17 (2° sem.), 40 (1908).
[22] G. Buglia, Bioch. Z., "Hamburger's Festband," p. 354, 1908.

serum with water produces an increase in surface tension until that point is reached at which an opalescence is produced. At this point the surface tension is lowered somewhat. This slight lowering of the surface tension and production of turbidity is observed even when the serum is dialyzed in a closed apparatus to prevent dilution.[23] The decrease in surface tension upon the addition of NaOH is probably due to the fact that the protein suspension dissolves with the transformation of inactive protein particles into active molecules. The lowering that is observed during dialysis therefore is probably brought about by the partial transformation of the protein ions into non-dissociated molecular protein, due to the elimination of one part of alkali with which the serum proteins have combined in the form of salts electrolytically dissociated.

Quagliariello [24] has observed that perfectly limpid and optically clear hemocyanin solutions, when the reaction of the solution is sufficiently far from the isoelectric point, become more opalescent as one approaches this point, where flocculation occurs. Probably if determinations of the surface tension had been made it would have been found that the maximum lowering occurs at the point of opalescence.*

Serum globulin of ox blood dialyzed for several months, washed with distilled water, dried in a current of hot air, pulverized, extracted with ether and finally suspended in water, does not show any great decrease in surface tension. But the same globulin dissolved in N NaOH affects the surface tension considerably as indicated by the experiment, the results of which are shown in Table 3: [25]

TABLE 3.

Distilled water (28° C.).	55 drops
Globulin suspension in distilled water	56½ "
Solution of the same globulin in N NaOH (reaction very alkaline)	66½ "
To the filtrate of the globulin suspension was added 5 drops of N NaOH (reaction very alkaline)....................	55½ "

The above experiment indicates that pure serum globulin does not give up any substances to water that by themselves or by the addition of NaOH affect the surface tension; whereas the addition of NaOH to the suspension notably lowers the surface tension indicating solution of the globulin.

Iscovesco [26] has observed that solutions of many hydrophilic colloids (gum arabic, starch) have a lower surface tension than that of water and that when the surface tension is greater this is due to the presence of salts. In fact, eliminating these by means of dialysis, the surface tension of the colloidal solution should be lowered. According to Zlobicki [27] the so-called colloidal solutions of starch and certain gums have a greater surface tension than that of water. This accords with the results obtained by Bottazzi with purified glycogen.

It is of interest that Iscovesco observed "that egg albumin diluted with 3 vol. of distilled water, shows a higher surface tension than that of water.

[23] Fil. Bottazzi, *Arch. Fisiol.,* **7,** 604-605 (1909).
[24] G. Quagliariello, "Chemical and physico-chemical researches on hemocyanin." Note 2. Colloidal properties and isoelectric point of hemocyanin. *Atti R. accad. med-chir. Napoli,* **74** (1920).
* See paper by E. O. Kramer in "Colloid Symposium Monograph, Vol. IV," Chemical Catalog Co., New York, 1927. *J. A.*
[25] Fil. Bottazzi, *Rend. accad. Lincei* (5), **21** (2° sem.), 226 (1912).
[26] H. Iscovesco, *Compt. rend. Soc Biol.,* **69,** 491, 537, 566, 622 (1910).
[27] Freundlich, "Kapillarchemie," p. 394, Leipzig, 1909.

and still more striking are his observations that dialyzed pure egg albumin powerfully increases the surface tension of water; whereas the globulin mixed with the egg albumin should lower it." [28] Iscovesco concludes that "this classical notion (which holds that albumins lower the surface tension of water) is absolutely erroneous in so far as egg albumin is concerned." [29]

Iscovesco's results have not been confirmed by Berczeller,[30] while Quagliariello [31] has recently demonstrated that egg white (crystalline ovalbumin) and also ovoglobulin lower the surface tension of water. The opposite results of Iscovesco were probably due to the fact that he worked with suspensions of ovoproteins rather than with true solutions.

Dialysed hemoglobin suspensions [32] behave similarly to those of serum globulin suspensions. They have a viscosity and surface tension scarcely different from that of water; but upon the addition of HCl or of NaOH the dispersed phase passes gradually into a state of solution (as hemoglobin chloride or sodium hemoglobinate), the viscosity increases and the surface tension is lowered as the liquid becomes clear. Upon the gradual addition of an excess of acid, the viscosity reaches a maximum and then diminishes. Under these conditions the surface tension remains low when the viscosity is much diminished. Neutralization of acid by base or vice versa, causes the clear liquid to grow turbid because the hemoglobin precipitates and the surface tension which had been lowered begins to increase. In proof of the above statements the following experiments of Bottazzi are of interest.

TABLE 4 *Experiment.*[*]

Water value of stalagmometer at 23° C. = 40.5.

Solution-suspension of dialysed hemoglobin (methemoglobin).

	Drops of 0.2N NaOH Solution Added	Number of Drops (Stalagmometer)	Temperature and Observations
Solution-suspension 10 cc.	0	44.0–44.5	23° C.
	1	44	"
	1	43.8–43.5	"
	1	43.6–44.6	"
	1	44.8–44.9	Liquid turbid
	1	45.2–45.3	" "
	1	45.5–46.3	" "
	1	46.4	" "
	1	46.6–46.8	Liquid redder-turbid
	1	47.0–47.5	" " "
	1	47.5–47.8	" " "
	1	47.5–47.8	Liquid clearer
	2	47.5	" "
	3	47.8–48.2	Liquid limpid
	3	47.0	" "
	Same (after 2 hours)	47.8	" "

* *Loc. cit.*, experiment III, pp. 264-265.

[28] Bottazzi, "Physikalisch-chemische Untersuchung des Harns und der anderen Korperflüssigkeiten," in C. Neuberg's "Der Harn, etc.," p. 1716, Berlin, 1911.
[29] H. Iscovesco, *Compt. rend. Soc. Biol.*, 69, 622 (1910).
[30] L. Berczeller, *Biochem. Z.*, 53, 232 (1913).
[31] G. Quagliariello, *Rend. accad. Lincei* (5ª), 31 (2° sem.), 120 (1922).
[32] Fil. Bottazzi, "The colloidal properties of hemoglobin." The effect of HCl and NaOH upon the viscosity and surface tension of suspensions of methemoglobin," *Rend. accad. Lincei* (5ª), 22 (2° sem.), 263 (1913).
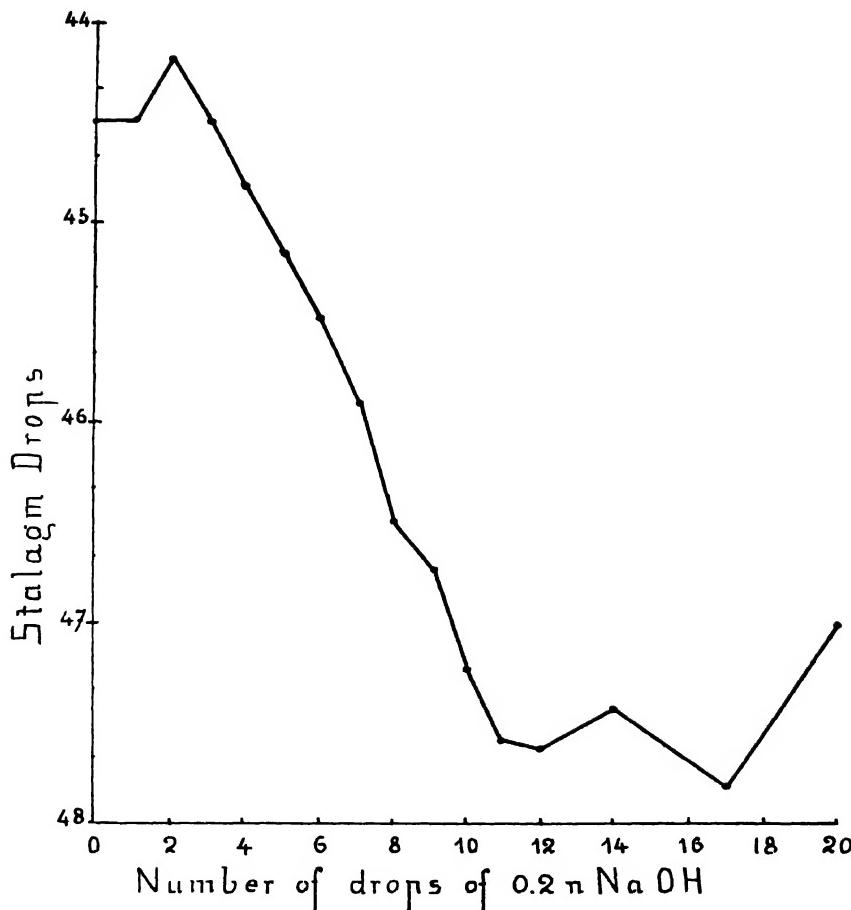
FIG. 2.—Graph of Results in Table 4.
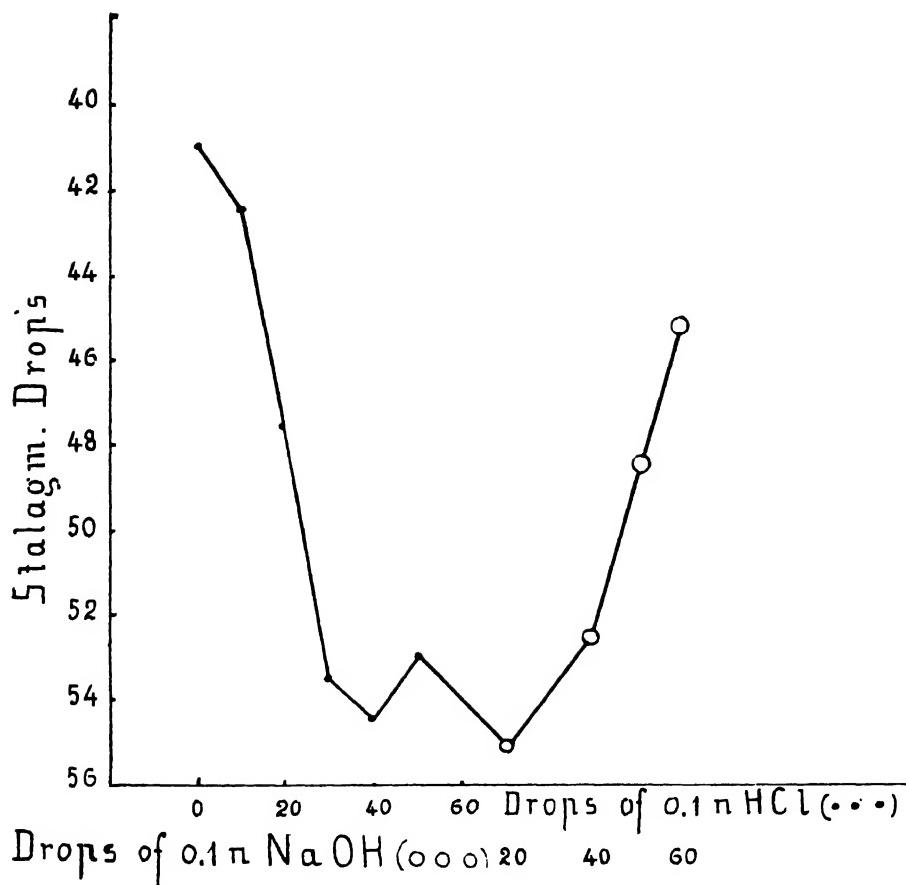
FIG. 3.—Graph of Results in Table 5

TABLE 5. *Experiment.**

Water value of stalagmometer at 23° C. = 40.5.

Water value of viscosimeter at 23° C.

Time (t) = 1 min. 17¾ sec.

	Drops Added 0.1N HCl or NaOH Solution	t 23° C.	Number of Drops (Stalagmometer)	Observations
Suspension of hemoglobin 10 cc.	0	1′ 18″	40.5	Liquid turbid
	10 0.1N HCl	1′ 22″	42.2	" "
	10	1′ 49⅓″	47.4	Liquid clear
	10	1′ 37⅖″	53.5	" "
	10	1′ 31½″	54.2	" "
	10	1′ 25″	52.5	" "
	20 0.1N NaOH	1′ 21½″	54.6	Increasing precipitation
	20	1′ 18″	52.2	Increasing precipitation
	10	1′ 18″	48.3	Increasing precipitation
	5	......	45 0	Increasing precipitation

* *Loc. cit.*, experiment IV, pp. 266 267

TABLE 6.

Animals	σ at 20° C.	Protein N Per Cent	Specific Gravity	Dry Residue Per Cent of Liquid	Ash Per Cent of Liquid	Ash Per Cent of Dry Residue
Scyllium stellare .	0.644	0.6772	1.028	6 327	1.755	27.74
Porco (Pig)	0.650	1 2300	. .	....	....	....
Coniglio (Rabbit)..	0 658	0 8384	. .	....	....	....
Pollo (Chicken) ...	0 660	0.6084	....	....	. .	....
Anatra (Duck) ...	0 665	0 5680	. .	....	....	....
Cane (Dog)	0 670	0 9512	. . .	....	....	....
Octopus macropus..	0.682	1.6599	1 052	12.03	2.971	24.71
Conger vulgaris ...	0 684	0.6034	. .	....	. . .	....
Rana (Frog)	0.692	0 6034	. .	....	. . .	....
Homarus vulgaris..	0 701	0 5277	1 033	4.334	3.599	83.03
Aplysia Limacina ..	0 754	0 0089	1 029	4.064	3.032	74.61
Sipunculus nudus ..	0.787	0.0089	1.026	....	....	....
Aplysia depilans ..	0 804	0.0089	. . .	. .	....	....
Holothuria Poli ...	0 935	0.0089	1 028	4.091	3.421	83.60
Aqua di mare* (from the basins of the Naples Zoological Station) ..	0 982– 1.003	. . .	1 029– 1 037	4.264	3.185	....

* Sea water.
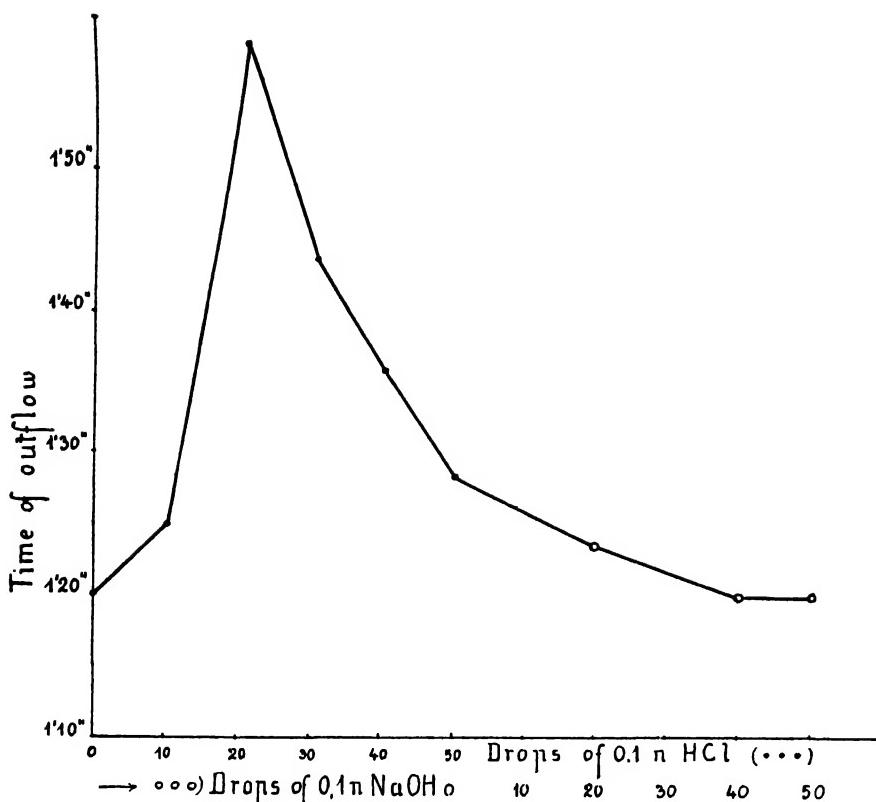
Fig. 4.—Graph of Results in Table 5.

Finally, it must be remembered that the surface tension of myosin suspension is decreased as the granules swell and dissolve upon the addition of KOH or HCl or lactic acid, and that in producing this effect potassium hydroxide is most effective; lactic acid least so.[55]

Another proof of the effect of proteins in natural solution is given by comparative determinations of the surface tension of blood (or of blood serum) of various vertebrates and invertebrates. From Table 6 in which data are reproduced from tables 75 and 103 of Bottazzi's monograph "Physikalisch-chemische Untersuchung, etc." (*loc. cit.*, p. 1659 and 1725), it follows that the greater the percentage content of protein substances of the blood or blood serum of various animals, the lower the surface tension. Small differences are due to the fact that the determinations of protein nitrogen and of surface tension do not refer to blood or serum of the same animals—having been made at different times. It is to be noted that with invertebrates the lowering of surface tension is not due to a non-protein substance, because the blood of these animals is in general poor in organic substances (fats, lower

[55] Fil. Bottazzi and E. d'Agostino, "Viscosity and surface tension of suspensions and solutions of muscle proteins under the influence of acid and alkali," *Rend. accad. Lincei* (5ª), 22 (2° sem.), 183 (1913).

fatty acids, etc.) capable of lowering the surface tension of water. The sudden increase in the surface tension from that of lobster blood rich in protein (protein $N = 0.5277\%$) to that of Aplysia blood lowest in protein (protein $N = 0.0089\%$) ranges from $\sigma = 0.701$ to $\sigma = 0.754$. The marine invertebrates whose blood is poorest in protein give the greatest values for σ, while those whose blood is rich in protein—Octopus, Homarus—have a relatively low value for σ. Blood serum of Scyllium has a very low value for σ (lowest of all), but its percentage content of protein is not proportionately great. In this case it is probably urea that lowers the surface tension of the serum. In fact Herlitzka [34] has demonstrated that the addition of urea to Ringer's or to Locke's solution lowers the surface tension:

TABLE 7.

Physiological Salt Solution	Surface Tension Dynes
Solution of NaCl and NaHCO₃	73.87
Ringer's Solution	73.54
Locke's Solution	73.39
Ringer's Solution with Urea	71.98
Locke's Solution with Urea	72.57

It is evident, therefore, that notwithstanding the addition of urea, the surface tension of perfusion liquids always remains greater than that of the blood serum of vertebrates which ranges from a maximum of 62.00 to a minimum of 52.28 dyne/cm. at 39°-42.5° C., as the results show in Table 8 from Fano and Mayer: [35]

TABLE 8.

Animals *	Temperature	Surface Tension in Dyne/Cm.
Cattle (7)	39° C.	61.00-62.00
Dogs (3)	39° C.	58.00-58.30
Guinea Pigs (2)	39° and 39.2° C.	59.00-62.00
Rabbits	39° " 39.5° C.	58.22-57.98
Women (2)	39° " 37° C.	58.20-59.89 and 59.77-60.00
Turkeys (3)	39° " 42.5° C.	52.60-56.00 " 49.50-53.75
Pigeons (4)	39° " 42° C.	52.60-56.00 " 49.50-53.75
Chickens (3)	39° " 42.5° C.	54.77-54.05 " 52.10
Goose	39° " 42.5° C.	52.28 " 49.39
Testudo graeca	23.5° " 39° C.	66.51 " 58.11
Tinca vulgaris	20° " 39° C.	63.99 " 57.60

* The numbers in parenthesis indicate the number of experiments in each case.

"The surface tension of the blood serum of birds is the lowest thus far determined; this is not a consequence of the higher body temperature of these animals, for the lower surface tension of the blood serum of birds is also exhibited at lower temperatures." [36]

Fano and Mayer also make the following interesting observation: "The surface tension of the blood serum of any animal increases with drop in temperature, as is seen with water and with any other solution. At the same temperature, however, the surface tension of the blood serum of mammals and cold-blooded (vertebrates) animals (turtle and tench) are about alike.

[34] A. Herlitzka, *Arch. Fisiol.*, 8, 249 (1910).
[35] G. Fano and M. Mayer, *Arch. Fisiol.*, 4, 164 (1907).
[36] Fil. Bottazzi, in C. Neuberg's "Der Harn, etc," p. 1726, Berlin, 1911.

If the temperature of the serum increases beyond the limit at which coagulation of the serum proteins usually begins, no anomalies in changes of surface tension are observed. Digestion and putrefaction reduce the surface tension of blood serum; the same result is observed if a certain quantity of its salt is removed from the serum." [36]

Evidently heating to the coagulation point does not produce anomalous variations. The influence of digestion and putrefaction is without doubt traceable to the activity of the products of protein hydrolysis. The salt effect has been mentioned above.

We are able to conclude, therefore, that protein substances are wholly inactive capillarily when they exist as microscopic or sub-microscopic suspensions, but that they lower the surface tension of water considerably when they exist in a state of solution. In support of the experiments indicated above, Bottazzi [37] in 1911 drew the following general conclusion, "that proteins in a state of perfect solution lower the surface tension of water, and that the reduction is, to a certain extent, proportional to their concentration." No protein was excepted. All of these substances (comprising gelatin, hemocyanin, lactalbumin, hemoglobin) are capillarily active. Furthermore, the first products of hydrolysis, the proteoses, seem to be more active.

THE PHYSICO-CHEMICAL CONDITIONS UNDER WHICH PROTEINS LOWER THE SURFACE TENSION OF WATER

We have seen that protein substances have the effect of lowering the surface tension of water. But proteins may exist under quite diverse physical-chemical conditions: they may exist as natural proteins in the normal body fluids, or precipitated or denatured, dialysed or crystallized and then redissolved or altered by heat.

The same substances may be obtained as protein salts of various acids, or of proteinates of the various alkali metals, etc.; they may be considered in the ionic state as positive or negative protein ions, and as undissociated compounds at the iso-electric point. Various authors have determined the relationships of osmotic pressure, viscosity, imbibition of water, electrical conductivity, electrical cataphoresis of various proteins under the conditions enumerated; but no one has studied the variations in surface tension that protein solutions present under these different conditions particularly when the protein exists as protein ion or as undissociated molecule.

Pauli [38] writes: "Neutral albumin shows slighter hydration or swelling, lower internal friction, lower osmotic pressure than ionic albumin. In contradistinction to electrically charged albumin, it is coagulable by alcohol and heat, and further only neutral portions of the substrate are precipitable by salts, acids and bases. By addition of small amounts of neutral salts, neutral albumin is stabilized against spontaneous flocculation, alcohol and heat coagulation, while ionically charged albumin is dehydrated and rendered susceptible to coagulating influences."

No mention is made of variations in surface tension.

According to Handovsky: [39] "The isoelectric point of the proteins is therefore that pH at which the number of protein ions is a minimum and the viscosity

[37] Fil. Bottazzi, in C. Neuberg's "Der Harn, etc.," p. 1719, Berlin, 1911.
[38] Wo. Pauli, "Kolloidchemie der Eiweisskorper," Part 1, p. 31, Dresden und Leipzig, 1920.
[39] H. Handovsky, "Allgemeine Chemie der Proteine," in C. Oppenheimer's Handb. der Biochemie, 2nd ed., Vol I, p. 555, Jena, 1924.

is likewise a minimum, the precipitability showing a maximum"; but even he neglects surface tension.

Even Loeb,[40] and Wo. Ostwald [41] neglect this point. Yet Wo. Ostwald draws attention (*loc. cit.*, p. 228) to observations of Buglia and of Frei: "Thus, e.g. the surface tension of neutral gelatin solutions and neutral blood sera is raised by small amounts of OH-ion, and depressed by H-ions (G. Buglia, W. Frei)." Handovsky (*loc. cit.*, p. 574) refers solely to Frei in the following: "The changes in surface tension of proteins by H-ions and OH-ions has been measured: OH-ions raise, H-ions lower the tension of gelatin and of blood serum against air (W. Frei)."

In order to understand the part played by Bottazzi and by his students in the development of the study of the surface tension of protein solutions and of the causes for their variation, it is necessary to remember: (1) that Buglia, at the time that he carried out these experiments, was an assistant of Bottazzi in Naples, and these and other researches of surface tension were made with the advice and under the direction of Bottazzi; (2) that the publications of the work of Buglia [42] and of that of Frei [43] were made in the same year (1908), but Frei published later; and, in fact, he cites the work of Buglia; (3) that Bottazzi first called attention to the results obtained by Buglia and Frei in his monograph: "Physikalisch-chemische Untersuchung des Harns und der anderen Körperflüssigkeiten" [44] in which a special chapter is devoted to surface tension in biology and medicine; (4) that, finally, researches were made successively by Bottazzi and his students in his laboratory, the result of which was a clearer understanding of the physico-chemical conditions under which proteins affect the surface tension of water.

Buglia (*loc. cit.*) observed that the addition of increasing quantities of various acids (hydrochloric, sulfuric, acetic) to blood serum, affects the surface tension in the sense that the curve presents two minima, the first less, the second more conspicuous; that following the first decrease the surface tension rises to its initial value, while after the second the curve "does not again rise to its original value, but runs for a long distance as an almost straight line parallel to the axis of abscissas. This characteristic of the curve has its counterpart in the behavior of the precipitate . . . which gradually redissolves, when the acid concentration is quite strong, and probably also in case the phenomena involved in the setting of the whole fluid mass have already begun, which are quite obvious with inorganic acids, but which on the other hand do not become very evident in serum with $C_2H_4O_2$ added to it [45]

Worthy of note is the fact that ". . . the depressions of each curve correspond to the formation in the serum of a flocculent precipitate, the return to the initial level to a re-solution of the precipitate, and the last portion of the curve to the commencement of the phenomena of setting of the whole fluid. The first barely perceptible drop, however, corresponds to only a very slight turbidity, observable only by careful comparison with clear normal serum" (*loc. cit.*, p. 325). And also important is this observation, that ". . . the precipitative action of hydrochloric acid on normal serum is less than that of

[40] J. Loeb, "Proteins and the theory of colloidal behavior," New York, 1922
[41] Wo. Ostwald, "Grundriss der Kolloidchemie." Sixth ed., Dresden u. Leipzig, 1921.
[42] G. Buglia, "Veranderungen der Oberflächenspannung des Blutserums unter dem Einfluss von verschiedenen Elektrolyten," *Biochem. Z*, 11, 311 (1908)
[43] W. Frei, *Transvaal Med. J.*, August, 1908. Idem., "Physical Chemistry and Veterinary Science," p. 4, Grahamstown, Cape Colony. 1908
[44] Fil. Bottazzi, in C. Neuberg's "Der Harn, etc.," pp. 1396-1761, Berlin, 1911.
[45] G. Buglia, "Veranderungen der Oberflächenspannung des Blutserums unter dem Einfluss von verschiedenen Elektrolyten," *Biochem. Z.*, 11, 311 (1908).

sulphuric acid, . . ." and that ". . . hydrochloric acid produces setting *in toto*, only when in high concentrations . . ." (*loc. cit.*, p. 326).

Examining now the results obtained by Frei: "By a series of investigations on gelatin solutions, W. Frei [46] found that the anions $SO_4^=$, Cl^-, NO_3^-
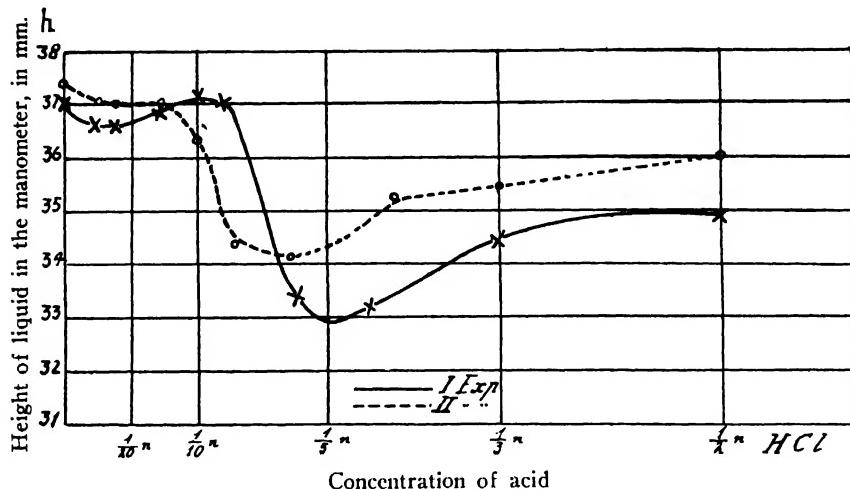
FIG. 5.—Surface tension changes on addition of HCl to blood serum.
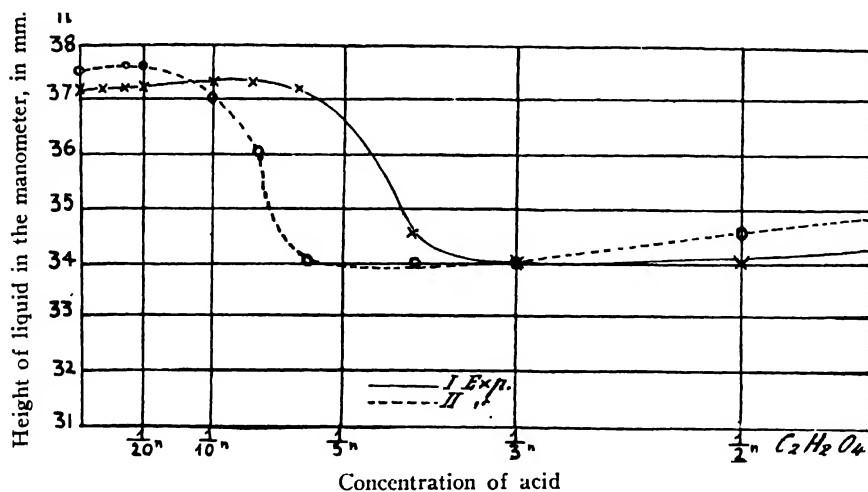
FIG. 6.—Surface tension changes on addition of acetic acid to blood serum.

raise the surface tension of neutral gelatin in the order given, while the cations Na^+, K^+, Mg^{++}, Ca^{++} raise it about in the same order as they increase the surface tension of water. The anions Cl^-, CO_3^-, NO_3^-, $SO_4^=$, $Acet.^=$ as Na-salts in $\frac{1}{6}$ n concentration lower in the following order: $CO_3^= > NO_3^- >$

[46] W. Frei, *Transvaal Med. J.*, August, 1908.

$SO_4^= > Cl^-$ the surface tension of a 1 per cent gelatin solution (excluding the acetate), if the solution is alkaline, they raise it (excluding the chloride) in the following series $Acet.^- > NO_3^- > SO_4^= > Cl^-$, if the solution is acid. The surface tension of neutral gelatin solution [and neutral serum] is raised by OH-ions, and lowered by H-ions.[47]

The determinations of pH of the original solution of gelatin not having been made, the results given are not easily interpreted. But with regard to the action of H^+ and OH^-, since the gelatin solution the author called neutral was certainly a solution of electro-negative gelatin, the action mentioned is found to accord perfectly with the theory which will be later expounded.

The same author found, moreover, "neutralization of the serum effects a material reduction in surface tension, and acidification (with H_2SO_4) powerfully lowers the surface tension of horse blood serum, whereas addition of alkali (KOH) raises it somewhat, but not proportionately to the concentration of OH ions." [48] Interesting also is the observation of Frei [49] that "the decrease in surface tension in grave cases of horse-sickness is probably due to the accumulation of CO_2 in the blood because it was possible to reproduce a similar decrease by an artificial imitation of this stage by strangulation of the jugular vein wherefrom the blood was taken."

The observations of Buglia and of Frei on blood serum coincide, therefore, perfectly and complement one another. They show that the surface tension of serum is not, under normal conditions, the possible minimum, that it is lowered, as with "neutral" gelatin, by the addition of acid (neutralization according to Frei) even twice successively (Buglia); and that succeeding the decrease there follows an increase with the increase in added acid (Buglia); that it is increased by the addition of alkali (Frei), as with gelatin; that when the surface tension reaches the first or second minimum, the serum becomes turbid, because it forms a precipitate which afterwards redissolves (Buglia); that finally the serum, following the addition of an excess of acid, after clearing ends by gelatinizing (Buglia).

Owing to the complexity of the chemical composition of serum and the presence in it of fats and lipoids as well as the salts of capillarily active organic acids, one might suspect that the lowering of surface tension were due to the liberation of a fatty substance, to the diminution of electrical dissociation of salts of organic acids, etc. But solutions of pure proteins behave in the same manner. One must admit, for this and for other reasons that will be given, that the lowering of surface tension of serum due to acids is dependent, at least in part, upon the serum protein. I might add, that the two minima observed by Buglia reveal the presence of at least two substances upon which acids act in the same manner and with the same effect, two substances characterized by different dissociation constants.

Bottazzi and his assistant d'Agostino [50] made the first researches upon the influence of the undissociated molecules and of the ions of pure protein substances upon the surface tension of water. They studied the surface tension of solutions of various concentrations (6.74, 3.41 and 1.705 per thousand) of serum albumin dialysed for a very long time (four months), and the influence of the addition of HCl and NaOH, using the stalagmometric method of Traube.

[47] Fil. Bottazzi, in C. Neuberg's "Der Harn, etc.," p. 1715, Berlin, 1911.
[48] Fil. Bottazzi, *loc. cit.*
[49] W. Frei, "Physical Chemistry and Veterinary Science," p. 5, Grahamstown, 1908.
[50] Fil. Bottazzi and E. d'Agostino, "On the surface tension of protein solutions," *Rend. accad. Lincei* (5ª), 21 (2° sem.), fasc. 9°, 3 Nov., 561 (1912).

TABLE 9. *Number of Drops of Solution of Serumalbumin of Constant Concentration After Adding HCl or NaOH.*

Serumalbumin of Constant Concentration 6.74 Per Thousand

Millimols HCl Added to 1 Litre of Solution	Serumalbumin Added per 1 Gram Millimols HCl	Number of Drops = n		Millimols NaOH Added to 1 Litre of Solution	Serumalbumin Added per 1 Gram Millimols NaOH	Number of Drops = n
0.00	0.00	57.8		0.00	0.00	58.1
2.29	0.34	58.0		1.15	0.17	55.4
3.43	0.51	59.1		2.88	0.43	54.7
5.41	0.80	59.1		4.56	0.68	54.8
6.81	1.01	58.9		6.54	0.97	55.0
8.49	1.26	59.0		9.60	1.42	55.4
10.2	1.51	57.4		14.6	2.17	56.4
15.7	2.33	58.3		21.1	3.13	58.3
18.9	2.80	58.9		38.1	5.65	61.8
27.4	4.07	61.6		52.2	7.75	63.3
52.2	7.75	64.3		164.0	14.2	65.9
95.8	14.2	66.0		237.0	24.3	68.4
164.0	24.3	67.5		303.0	35.2	69.7
237.0	35.2	68.4			45.0	70.7
303.0	45.0	69.1				

Serumalbumin of Constant Concentration 3.41 Per Thousand

Millimols HCl Added to 1 Litre of Solution	Serumalbumin Added per 1 Gram Millimols HCl	Number of Drops = n		Millimols NaOH Added to 1 Litre of Solution	Serumalbumin Added per 1 Gram Millimols NaOH	Number of Drops = n
0.00	0.00	56.6		0.00	0.00	56.7
1.26	0.40	57.6		1.81	0.53	54.2
1.77	0.52	57.1		3.61	1.06	54.2
3.50	1.03	55.6		5.39	1.58	54.3
5.23	1.53	54.6		7.16	2.10	54.6
6.96	2.04	54.6		8.94	2.62	54.7
8.66	2.54	54.8		12.4	3.64	54.9
10.4	3.05	54.9		17.6	5.16	55.7
18.7	5.48	55.9		27.7	8.12	57.3
52.8	15.5	59.6		75.6	22.2	60.9
141.0	41.4	62.6		150.0	44.0	63.4
290.0	85.0	65.1		229.0	67.2	64.8
				298.0	87.4	65.6

Serumalbumin of Constant Concentration 1.705 Per Thousand

Millimols HCl Added to 1 Litre of Solution	Serumalbumin Added per 1 Gram Millimols HCl	Number of Drops = n		Millimols NaOH Added to 1 Litre of Solution	Serumalbumin Added per 1 Gram Millimols NaOH	Number of Drops = n
0.00	0.00	55.4		0.00	0.00	55.6
0.58	0.34	56.9		1.16	0.68	54.2
1.16	0.68	56.0		3.18	1.87	54.2
1.74	1.02	55.3		4.90	2.87	54.4
3.18	1.87	54.3		8.59	5.03	54.7
8.87	5.20	54.7		14.2	8.33	55.5
14.7	8.63	55.3		27.7	16.3	56.9
26.7	15.7	55.6		52.8	31.0	58.3
52.8	31.0	58.7		96.6	56.8	60.7
96.6	56.8	60.4		166.0	97.4	62.3
166.0	97.4	62.0		239.0	140.0	63.9
239.0	140.0	63.4		304.0	178.0	65.4
304.0	178.0	64.5				

TABLE 10. *The Influence of Sodium Chloride in Moderate Concentration.*
All serumalbumin solutions have a constant concentration of 1.25 per thousand.

Without NaCl				NaCl of Constant Concentration of 75.8 Millimols per Litre				NaCl of Constant Concentration of 22.7 Millimols per Litre			
Millimols of HCl Added to 1 Litre of Solution	Number of Drops = n	Millimols of NaOH Added to 1 Litre of Solution	Number of Drops = n	Millimols of HCl Added to 1 Litre of Solution	Number of Drops = n	Millimols of NaOH Added to 1 Litre of Solution	Number of Drops = n	Millimols of HCl Added to 1 Litre of Solution	Number of Drops = n	Millimols of NaOH Added to 1 Litre of Solution	Number of Drops = n
0.00	56.9	0.00	56.9	0.00	58.0	0.00	58.1	0.00	57.4	0.00	57.2
0.90	57.6	1.00	54.3	1.00	60.1	0.83	57.6	0.50	58.4	0.33	56.4
2.64	57.4	2.64	54.4	1.98	61.5	1.98	56.9	0.99	59.0	0.99	56.0
3.94	55.9	5.21	54.6	2.96	63.6	2.96	57.2	1.64	59.4	1.48	55.9
6.18	55.0	15.7	56.3	3.94	65.1	3.94	57.6	2.21	60.1	2.13	55.7
8.06	55.3	30.6	58.8	5.05	65.3	5.86	58.2	2.61	60.6	3.72	55.8
10.2	55.8	53.3	60.4	6.01	65.0	11.4	59.8	3.25	61.3	5.57	56.0
15.7	57.1	88.8	62.5	7.13	64.8	29.6	61.9	3.78	61.5	9.27	56.6
29.6	59.7	133.0	64.4	8.06	64.8	53.3	63.3	4.18	61.4	14.8	57.4
53.3	61.6	....	....	9.93	64.4	133.0	65.6	4.81	61.1	26.6	58.9
88.8	63.6	....	....	12.1	64.3	....	....	5.88	60.5	44.4	59.7
133.0	65.4	....	....	16.2	64.3	....	....	7.53	59.9	66.6	60.6
....	....	....	....	29.6	64.4	....	....	8.26	59.9	....	....
....	....	....	....	53.2	65.0	....	....	9.27	59.8	....	....
....	....	....	....	133.0	67.0	....	....	11.1	59.8	....	....
....	....	....	....	....	....	....	....	14.8	60.1	....	....
....	....	....	....	....	....	....	....	26.6	61.0	....	....
....	....	....	....	....	....	....	....	44.4	62.4	....	....
....	....	....	....	....	....	....	....	66.6	63.3	....	....

TABLE 11. *Influence of Sodium Chloride in Small Concentrations.*
(All the solutions contain 1.25 per thousand of serumalbumin; the data are corrected to temperature of 24° C.)

Without NaCl		NaCl of Constant Concentration: 1.25 Millimols per Liter	
Millimols HCl Added to 1 Liter of Solution	Number of Drops = n	Millimols HCl Added to 1 Liter of Solution	Number of Drops = n
0.00	55.17	0.00	55 56
0.25	57.58	0.41	57.89
0.41	57.44	1.07	56 44
0.50	57.36	1.80	54.80
0.83	55.78	2.45	54.50
1.64	54.60	3.09	54.50
2.13	54.20	4.34	54.50
2.41	54.20	6.78	54.70
3.25	54.20	10.7	55.33
5.11	54.30	18.6	56.33
7.41	54.50	26.6	57.11
14.8	55.44	....	....
26.6	56.89	....	....

TABLE 12. *Influence of NaCl in Small Concentrations.*
(All the solutions contain 1.25 grams of serumalbumin per liter; the data
are corrected to a temperature of 24° C.)

Without NaCl		NaCl of Constant Concentration: 4 Millimols per Liter	
Millimols HCl Added to 1 Liter of Solution	Number of Drops = n	Millimols HCl Added to 1 Liter of Solution	Number of Drops = n
0.000	55.40	0.000	55.70
0.075	55.60	0.087	56.30
0.162	56.67	0.174	57.03
0.248	56.89	0.296	57.59
0.333	57.22	0.394	58.14
0.430	56.89	0.490	58.26
0.728	55.30	0.586	57.92
1.19	54.60	2.03	55.80
1.74	54.10	2.27	55.40
2.27	54.10	2.68	55.30
4.17	54.30	3 26	55.30
7.14	54.50	4.17	55.30
12.5	54.90	25 0	57.48

These experiments were made at a temperature varying from 23° to 25 5° C,
which interval of temperature produced a variation in the distilled water of
only one third of a drop. At 24° C. the water value for the stalagmometer
was 54.1 drops.

They carried out two series of experiments: (1) In the first they de-
termined the variations of the number of drops *n* of solution of serum
albumin, maintaining the concentration of albumin constant, and varying the
concentrations of hydrochloric acid or of caustic soda; (2) in the second series
they maintained constant the concentration of albumin and of sodium chloride,
varying the concentrations of hydrochloric acid or of caustic soda.

The values in Tables 9 and 10 were obtained with serum albumin from
ox blood and those in Tables 11 and 12 with serum albumin of ox blood
identical with the preceding, and treated in the same way, but dialysed in
another dialyser.

Figures 7 and 8 correspond to Table 9; Figures 9, 10 and 11 to Tables 10,
11 and 12.

The three curves (*see* Fig. 8) *ABC*, *abc*, *αβγ* correspond respectively to
the three different concentrations of serum albumin already referred to:
6.74 per thousand, 3.41 per thousand and 1.705 per thousand.

The number of drops *n* given by the original solution of serum albumin
is indicated at the value 0 (zero) on the abscissa.

The acid zone lies to the left of zero: that is to say in that zone values of *n*
are indicated as HCl, in increasing amounts from 0 to 5 millimols of acid per
gram of serum albumin, is added. Similarly, values are indicated for *n* in the
alkaline zone to the right with corresponding additions of NaOH.

Each of the three curves has a maximum for *n* in the middle (*A*,*a*,*α*), a mini-
mum in the acid zone (*B*,*b*,*β*) and another minimum in the alkaline zone (*C*,*c*,*γ*).
Having passed the two minima, each of the three curves rises towards a new
maximum that is not indicated in Figure 8 (*see* Table 9 and Fig. 7). It will
be noted that all three median maxima for the three curves fall in the acid zone
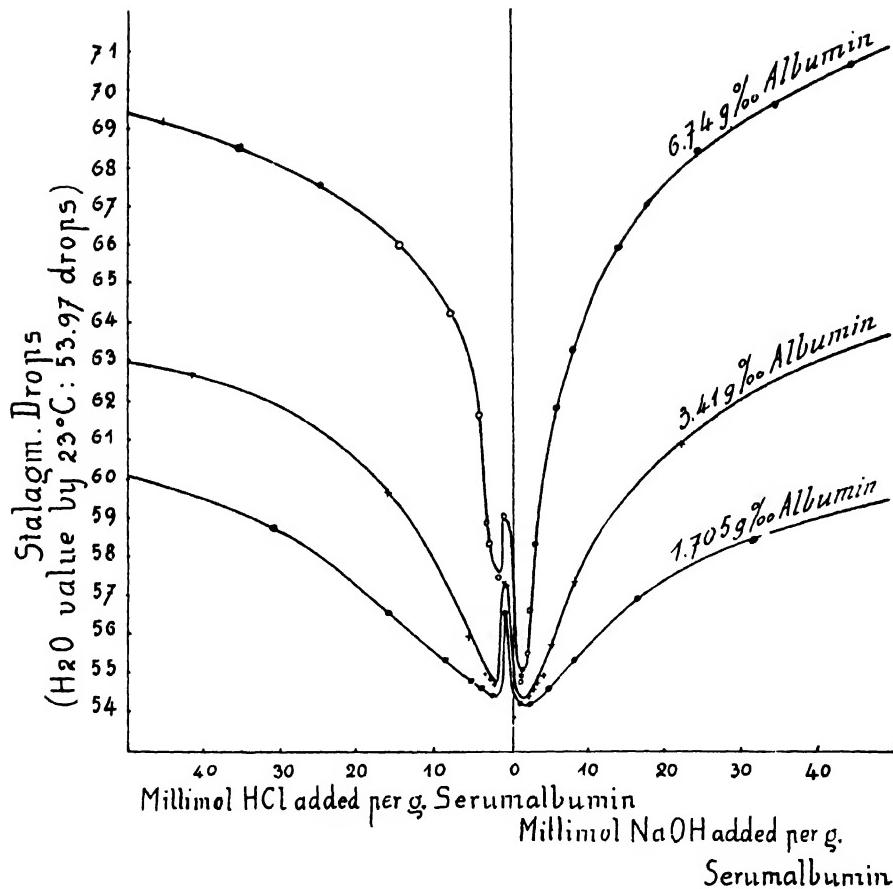
Fig. 7.
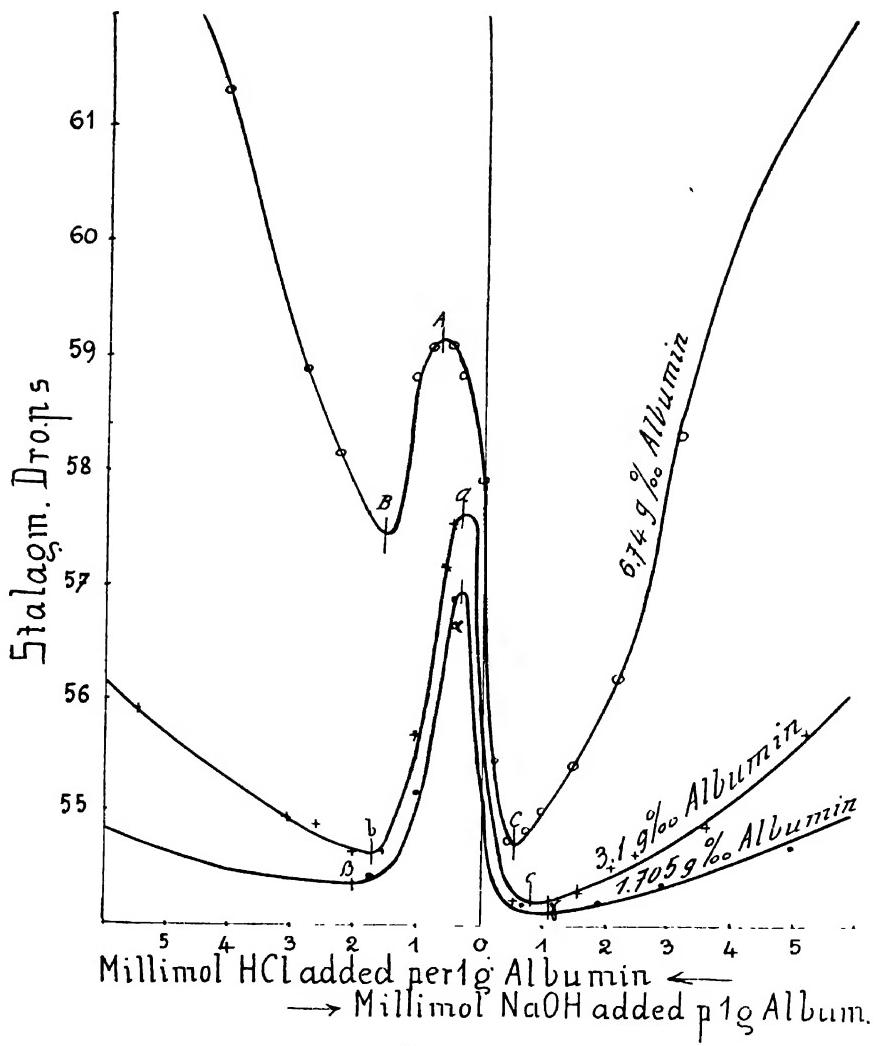
Fig. 8.
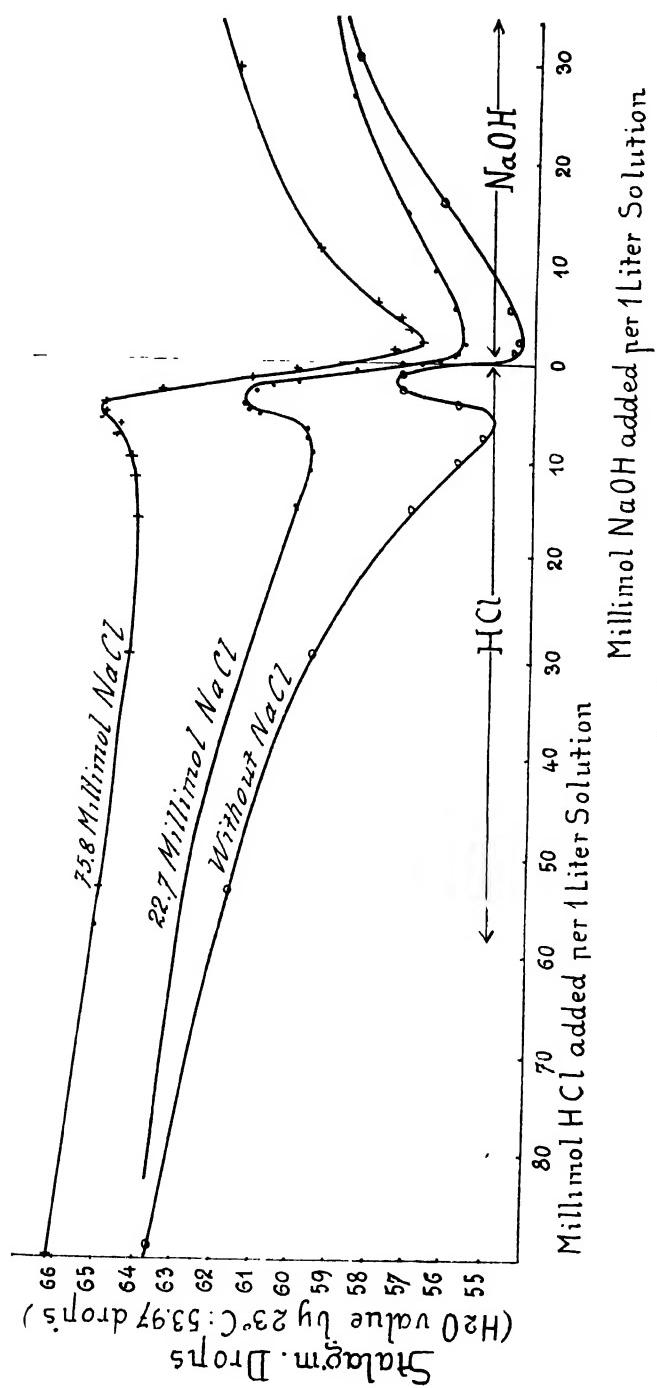
FIG. 9.
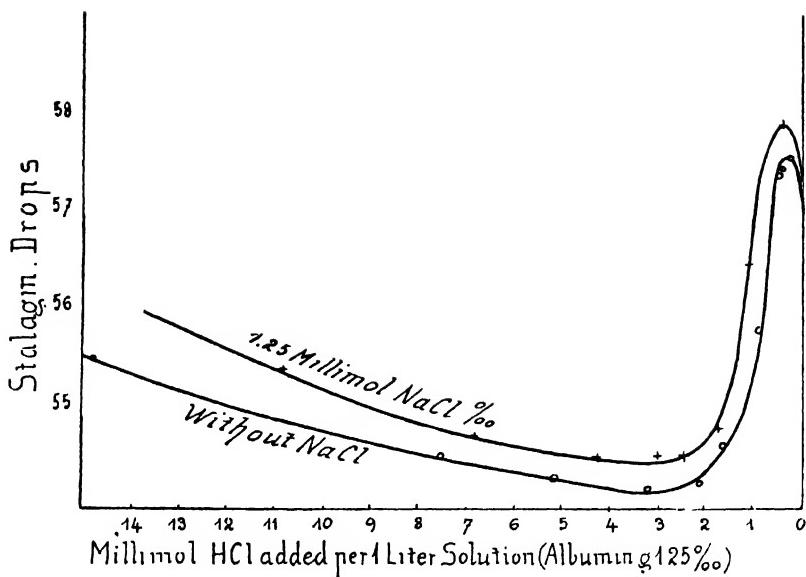
FIG. 10.
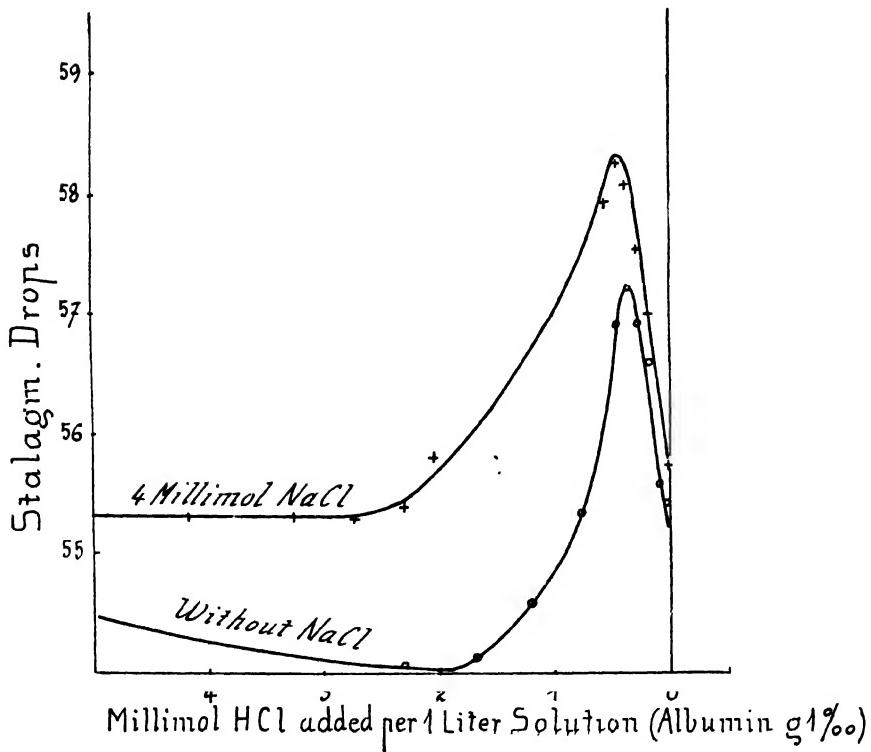
FIG. 11,

The values for *n* for the three original solutions:

For solution 6.74 per thousand................. $n = 57.8$-58.1
"   " 3.41 per thousand................. $n = 56.6$-56.7
"   " 1.705 per thousand................. $n = 55.4$-55.6

differ little from that of distilled water ($n_{24°} = 54.1$), but are notably greater for the more concentrated solution, that is, increase with the concentration of serum albumin.

Following the addition of acid, the number of drops increases to a maximum (A,a,α), then diminishes to a minimum (B,b,β), finally increases and surpasses by far the first maximum. This shows that the addition of acid first lowers the surface tension, then increases it, and finally lowers it anew.

Following the addition of alkali, however, *n* first diminishes, reaching a minimum (C,c,γ), and then increases to a maximum not indicated in the graph. That is to say, alkali first increases the surface tension of solutions of serum albumin and then lowers it.

The explanation of this curve in the work of Bottazzi and d'Agostino (p. 568) appears to be that with an increase of *n* (that is a diminution of the surface tension of the solution) there corresponds an increase in molecular concentration of undissociated protein. In the original solution the pure serum albumin is in part dissociated as acid, in fact, migrates towards the anode under the influence of an electrical field. The first small quantities of acid added decrease such dissociation, and carry the albumin to the isoelectric point (A,a,α) where it manifests its maximum capillary activity and minimum surface tension. Increasing the concentration of acid (from A,a,α to B,b,β) forms albumin chloride with a maximum electrical dissociation at B,b,β. To the increasing ionization of the albumin there corresponds an increase in surface tension indicated by a fall of the three curves. To the maximum ionization there corresponds a minimum capillary activity of the albumin B,b,β. This value of surface tension is greater than that of the original protein solution, which demonstrates that in this the albumin is less ionized than corresponds to the points B,b and β.

Increasing then the concentration of the acid the surface tension of the solution becomes lower. Why? One may admit that acid reduces the dissociation of albumin chloride. With this hypothesis, however, one can perhaps explain one part of the lowering; but the further lowering of the surface tension is not readily explained. Probably this is due, at least in part, to hydrolysis of the protein, that is, to the lowering action of the first products of hydrolysis.

I have said that pure albumin is in small part dissociated as acid. The addition of NaOH at once produces sodium albuminate which is strongly dissociated, whence are formed many electronegative protein ions, which raise the surface tension to a maximum. The further addition of NaOH causes a considerable progressive lowering of surface tension, owing to some extent to the decreasing dissociation of sodium albuminate caused by the presence of an excess of sodium ion. Another factor in causing this might be the hydrolysis of the protein.

A study of the experimental results collected in the tables and charts suggests the following general considerations.

In order to carry the albumin from the state of minimum dissociation

at points *A*, *a*, and *α* to a state of maximum dissociation of chloride at points *B*, *b*, and *β*, the following amounts of HCl are necessary (Fig. 8):

TABLE 13.

	Concentration of Albumin Per Thousand	Millimols HCl per Gram Albumin
1st Solution	6.74	0.9
2d Solution	3.41	1.4
3d Solution	1.705	1.7

The following amounts of NaOH are necessary to bring about the maximum dissociation of the albuminate—points *C*, *c* and *γ* (Fig. 8):

TABLE 14.

	Concentration of Albumin Per Thousand	Millimols NaOH per Gram Albumin
1st Solution	6.74	1.1
2d Solution	3.41	1.15
3d Solution	1.705	1.3

The more dilute the solution, therefore, the greater the quantity of acid or of base is required to carry the albumin salt to a state of maximum dissociation. The explanation of this is obvious if one considers the characteristic properties of these salts whose acid or basic components constitute a weak electrolyte, such as, for instance, potassium cyanide. The hydrocyanic acid being but feebly dissociated, solutions of potassium cyanide are notably alkaline, because a certain amount of the salt is split off forming KOH and undissociated hydrogen cyanide. This increases with the dilution. In fact Shields [51] has found experimentally the results shown in Table 15.

TABLE 15.

Molecular Concentration of KCN	Degree of Hydrolysis
0.947................	0.31
0.235......	0.72
0.095..........	1.12
0.024..................................	2.34

In our case the degree of hydrolysis should be more apparent, because the concentrations of protein salts were so small (probably less than 1 millimol of albumin per liter).[52]

In order to depress the hydrolysis of potassium cyanide and to carry the salt to a state of maximum dissociation, it is necessary to add an excess of potas-

[51] Z. physik. Chem., 12, 167 (1893).
[52] Calculating the hydrolysis of the protein salts from research of d'Agostino and Quagliariello (Nernst-Festschrift-Band, Z. physik Chem., p. 27, 1912), for the three concentrations used in our experiments, the following values are found:

Concn. sodium albuminate	6.74 g. albumin per thousand degree of hydrolysis	6.9 per cent
" " "	3.41 g. " " " " " "	9.6 " "
" " "	1.705 g. " " " " " "	13.2 " "
Concn. albumin chloride	6.74 g. " " " " " "	1.81 " "
" " "	3.41 g. " " " " " "	2.53 " "
" " "	1.705 g. " " " " " "	3.56 " "

sium hydroxide, wherefore at the state of maximum dissociation of KCN, equivalent quantities of HCN and KOH are no longer present, since KOH is present in excess.

In our case the quantity of NaOH or of HCl amounts to about one millimol per one gram of albumin, or one mol per one thousand grams of albumin. (Consequently the molecular weight of albumin must be greater than one thousand.)

Bottazzi draws the following conclusions in accord with general chemical principles:

(1) The quantity of acid or of base necessary to add in order to carry the albumin salt to a state of maximum dissociation increases with the dilution.

(2) The quantities of acid and of base are of the same order of magnitude.

(3) It is apparent that albumin has a molecular weight greater than one thousand.

To explain other characteristics of the curves, one must admit that albumin chloride of the same concentration is less dissociated than sodium albuminate. In other words albumin chloride is a feeble electrolyte, while sodium albuminate is a moderately strong electrolyte. In the more dilute solutions, then, the degrees of dissociation of the two salts do not differ greatly, while in more concentrated solutions the difference between the degrees of dissociation is notable. This accords perfectly with the principles of physical chemistry.

This hypothesis explains all the secondary properties presented in the curves. In fact, the feeble dissociation of albumin chloride affects all that portion of the curve found in the acid zone. The rise of the curve from the point A is slight, but it becomes gradually accentuated near point B with increasing concentration of albumin chloride. Therefore, the portions of the curve of the acid zone are less concave than those portions of the alkaline zone; and while the portions AC, ac and $a\gamma$ are near one another those portions AB, ab and $a\beta$ are distant from one another.

The facts just emphasized are more evident from researches made with a constant concentration of albumin and of sodium chloride (cf. Tables 10, 11, and 12 and the corresponding Figs. 9, 10 and 11). NaCl having common ions with both the protein salts diminishes their dissociation. But while this influence is striking enough with albumin chloride, it amounts to little with sodium albuminate. Hence, those portions of the curve of the acid zone are much raised and their concavity diminishes progressively with increasing concentrations of NaCl. Wherefore, the maximum influence is exerted on the completely dissociated chloride (points B) and is less at points A, the apices of the curves.

Bottazzi and d'Agostino's results of 1912 have been confirmed recently by Quagliariello,[53] Bottazzi's assistant, who by researches made upon solutions of dialysed hemoglobin has found in fact that various acids (lactic, hydrochloric) lower the surface tension of solution of dialysed hemoglobin as with solutions of other proteins. Wherefore it is apparent that such action is common to all acids and to all solutions of dialysed protein substances. "Acids acting upon protein salts as proteinates, or dissociated protein anions and hydrogen ions, drive these proteins to their isoelectric point, at the point, that is, of their minimum electrolytic dissociation; exactly at this point the lowering action of the protein reaches its maximum value.

[53] G. Quagliariello, "Influence of acids and alkalis upon certain physical-chemical properties of hemoglobin," *Arch. Science Biol.*, 2, 423 (1921).

"It follows, therefore, that different acids vary in their action as they are more or less dissociated; and that with a given acid the quantity necessary to add to cause the protein solution to reach its minimum value of surface tension is the greater the more distant the isoelectric point of the protein is from neutrality."

Quoting fully the researches of 1912 of Bottazzi and d'Agostino, Quagliariello says (*loc. cit.*, p. 426 *et seq.*): "These authors (Bottazzi and d'Agostino) adding HCl in increasing quantities to dialysed serum albumin, but in such a manner as to keep the concentration of albumin constant, found that the acid at first at a minimum concentration lowers the surface tension; then at a mean concentration increases it and finally at concentrations relatively high lowers it anew. The quantity of acid necessary to reach the minimum of surface tension is greater the greater the concentration of albumin.

"From these facts the authors make the following deduction: Serum albumin is an amphoteric electrolyte whose acid constant of dissociation is greater than its basic constant (according to Michaelis $\frac{K_a}{K_b} = 7 \times 10^4$). In a solution of serum albumin perfectly dialysed, the protein exists for the most part as undissociated molecules; to a slight extent as albumin anions and hydrogen ions.[54] If to such a solution one adds an acid, it first depresses the dissociation of the albumin [55] and since undissociated, molecular albumin is more efficacious in lowering the surface tension than albumin ions, it follows that the surface tension of the solution is lowered. But when the albumin has reached its isoelectric point each further addition of acid converts it to salt; the albumin that existed practically entirely as undissociated molecules passes to a state of albumin cations and the surface tension is increased; finally when all the albumin has been converted to salt a continued addition of acid tends to depress the dissociation of the albumin chloride because of the common ion (Cl^-), and forms anew undissociated molecules (this time of albumin chloride) which lowers the surface tension. The alkalies act in conformity with this interpretation forming albumin anions, first increasing the surface tension of the solution and then at a certain concentration lowering it, owing to the depression of dissociation of sodium albuminate by the common ions, and increase anew the number of undissociated molecules (this time of sodium albuminate).

"This simple interpretation implies, however, that the albumin exerts a lowering action when it is found in the molecular condition, while ionized its effect is much less. The experiments of Traube [56] and of Windisch and Dietrich [57] indicate that molecules are more efficacious in lowering surface tension of water than ions. For example, the following experiment is indicated by Michaelis.[58] Eucupine bichloride dissolved in water lowers its surface tension to a certain extent (for example a 1% solution gave 113 drops against 84 drops for the pure solvent). If alkali is added to the solution the surface tension falls to a minimum (in the experiment considered 187 drops). The

[54] This fact was discovered simultaneously but independently of each other by Pauli and Handovsky, by Bottazzi, and by Michaelis, in 1909 (See note 2, p 612 of the work of Bottazzi· Researches upon solutions of organic colloids. *Arch. Fisiol.*, **7**, 579 (1909).)
[55] On p. 622 of the work cited above, Bottazzi expressed himself thus, in 1909: "Acting in small amounts upon this so-called neutral albumin, that is to say, well dialysed, acids assist the thermal coagulation probably because they drive back the dissociation"
[56] *Biochem. Z.*, **42**, 470 (1912). *Intern. Z. physik. Chem. Biol.*, **1**, 10 (1914).
[57] *Biochem. Z.*, **97**, 135 (1919); *Ibid.*, **100**, 10 (1919).
[58] L. Michaelis, "Praktikum d. physik. Chemie insbesondere der Kolloidchemie für Mediziner und Biologen," Berlin, 1921.

explanation of the phenomenon is as follows. The depressing substance is eucupine, not the eucupine ion. Molecular eucupine is already present in small concentrations in the aqueous solution of the salt, because of hydrolysis, but in measure as the acidity of the solution diminishes, its concentration increases and hence the surface tension is lowered.

"Furthermore a solution of salicylic acid according to Berczeller [59] has a low surface tension, because of the undissociated molecules that it contains; while the addition of an acid inactive in itself produces a further lowering of surface tension.

"It is evident that Bottazzi and d'Agostino's conclusions regarding the
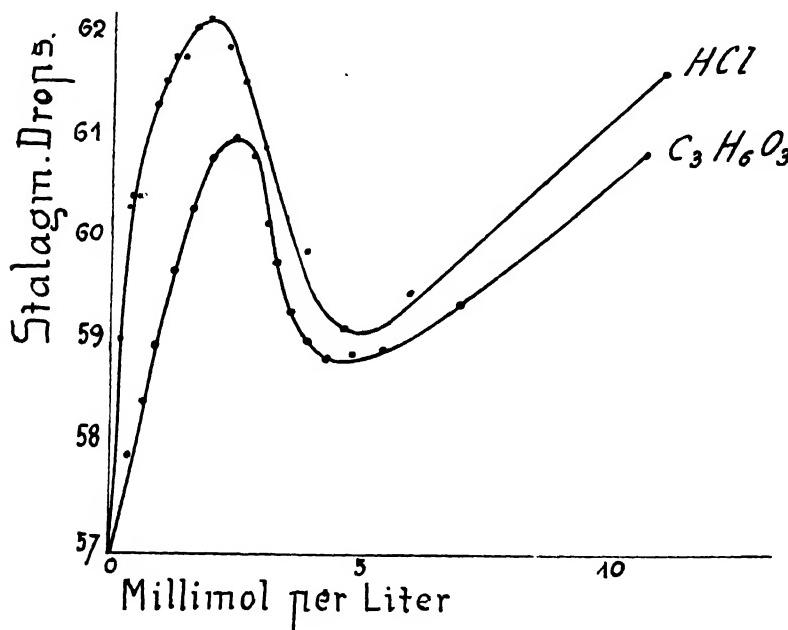
Fig. 12.—Surface tension changes in a hemoglobin solution.

action of acids upon the surface tension of protein solutions has the merit of simplicity. In keeping with these conclusions is the fact observed by Camis [60] that small amounts of lactic acid lower the surface tension of hemoglobin."

The curves in Figures 12 and 13 from the work of Quagliariello are exactly like those constructed by Bottazzi and d'Agostino. The first shows a lowering, the succeeding increase and the further lowering of the surface tension caused by the addition of HCl and of lactic acid in increasing amounts to a solution of hemoglobin. And the following figure shows the influence of HCl and of NaOH.

At the isoelectric point [61] proteins present a minimum value for surface

[59] *Biochem. Z.*, **53**, 238 (1913). *Intern. Z. physik. Chem. Biol.*, **1**, 124 (1914).
[60] M. Camis, *Haematologica*, **2**, 149 (1921). *Idem., Arch. Scienze Biol.*, **2**, 134 (1921).
[61] This refers with some slight modification to the reasoning of Quagliariello which is founded upon rtain observations and considerations of Bottazzi and d'Agostino made in 1912.

tension, because molecules are more efficacious than ions in lowering the surface tension of water; but in reality this is not an explanation; because the minimum surface tension and minimum ionic dissociation coincide, it does not necessarily mean that one is the effect of the other.

However, not only the surface tension, but also all the physico-chemical properties of proteins have what might be called a critical value at the iso-electric point. It is apparent from the researches of Pauli, Michaelis, Loeb and others that the solubility, osmotic pressure, viscosity, imbibition capacity all have their minimum value at the isoelectric point.

Now, if all these properties vary in the same sense with varying concentrations of hydrogen ions, it is probable that this arises from the same cause;
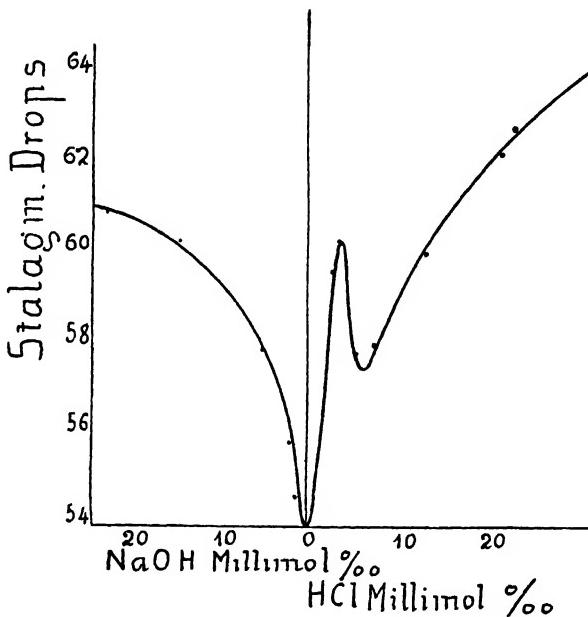
FIG. 13.—Surface tension changes in a hemoglobin solution.

and moreover with variation of the concentration of the hydrogen ion there is a corresponding variation in the degree of dissociation of the amphoteric substance. One must admit that all the properties considered above depend more or less upon the degree of dissociation of the amphoteric substance. The explanation generally accepted to-day for this fact is that the hydration of the protein ions (whether anion or cation) is notable, while that of the undissociated molecule (whether the pure substance or the salt) is slight. Now if we accept this theory we see why at the isoelectric point at which the ionic concentration is practically nil, stability, osmotic pressure, imbibition, viscosity may have minimum values; why away from the isoelectric point towards acidity or alkalinity, all these values increase until they reach the maximum which corresponds to the maximum degree of dissociation of the salt which is formed (maximum ionic concentration); and also why every further increase of acidity or alkalinity after reaching the maximum has the effect of

diminishing the values considered that correspond to a decrease in dissociation.[62] At the isoelectric point minimum ionic concentration may lead to molecular aggregation and precipitation. But while an aggregation at the isoelectric point is not necessary to explain the lowering of osmotic pressure, viscosity, etc., this is otherwise with surface tension. If a molecular aggregation, a formation of granules, would take place, there should be an increase and not a diminution in surface tension.*

That at the isoelectric point proteins present a maximum degree of instability or minimum degree of solubility is beside the point; this is simply the effect of their molecular condition. That at the isoelectric point most protein solutions become turbid and precipitate, passing from a state of solution to a state of suspension is quite true; but when this happens we see the values of surface tension of the solution along with those of osmotic pressure, viscosity, etc., rapidly approaching that of the pure solvent.

In solutions of relatively soluble proteins such as serum-albumin and hemoglobin, in relatively dilute solution such as we are considering, at the isoelectric point the surface tension is lowered, yet without apparent precipitation indicating molecular aggregation. In other words, at the isoelectric point there is possibly a molecular association that precedes the precipitation of a colloid, when this precipitation occurs; but when precipitation occurs the surface tension of the solution tends to increase. The precipitation of the colloid, which is always preceded by molecular association, is a secondary fact that depends upon the greater or less stability of the dispersed particles, and that in certain cases (especially globulin, hemocyanin) it is rapidly apparent when the isoelectric point is reached, while in other cases it takes place with great slowness or not at all (for example: serum albumin, hemoglobin). Proteins are characterized at their isoelectric point by their molecular condition (undissociated molecules): a partial or total precipitation (preceded by molecular aggregation) may occur, but it may fail, or take place with extreme slowness.

While I believe that at the isoelectric point one is always able to feel sure a certain degree of aggregation of the molecules, I deny that one is able to invoke the lowering of surface tension as the proof of such aggregation.

The pH of the liquids in which surface tension determinations were made was not determined either in my researches of 1912 or in those recently made by Quagliariello. In new researches made together with my assistant, Dr. de Caro, and already communicated in part to the International Congress of Physiology that met in Edinburgh in July of 1923,[63] I have examined the

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[62] It is questionable to hold that the maximum values of viscosity, osmotic pressure, etc., of a protein solution to which is added alkali or acid, correspond to the maximum degree of dissociation of the salt; and the diminution of such values represent a repression of dissociation owing to a common ion effect. From a theoretical point of view the objection might be raised that protein salts which are formed by the union of protein with a strong acid (as, for example, protein chloride), or with a strong base (as, sodium proteinate), are not greatly affected (Michaelis). From the experimental point of view Loeb has observed that while the osmotic pressure, etc., diminish by an excess of acid or alkali, the electrical conductivity which is a direct measure of ionic concentration continues to increase. Therefore, Loeb in a series of interesting researches published in the *Journal of General Physiology* and collected into one volume "Proteins and the Theory of Colloidal Behavior" (1922) seeks to explain the behavior of proteins on the basis of Donnan's membrane equilibrium. However that may be, what interests me here is that Loeb's curves for osmotic pressure, viscosity, imbibition, etc., of gelatin as a function of pH are very similar to those obtained by Bottazzi and d'Agostino and Quagliariello for surface tension. I will add that Donnan's membrane equilibrium has nothing to do with the phenomenon of surface tension.

* Aggregation may raise or may lower any of these properties, depending on whether it causes approach to or recession from the zone of maximum colloidality. See Vol. I, Chapter I. *J. A.*

[63] Fil. Bottazzi, "On Surface Tension of Protein Solutions," *Quarterly J. Exp. Physiol. Suppl.*, Volume, p. 65, London, 1923. See also *Giornale di Biologia e Medicina Sperimentale*, 1, fasc. 4*, August, 1923.

question from this point of view, upon the importance of which Loeb [64] has recently insisted.

In this new series of researches we have experimented with solutions of gelatin (the same gelatin recently used by Loeb, which he kindly sent me), and with albumin from ox blood well dialysed, after having found that serum globulin or pure casein did not give satisfactory results principally because these two proteins are insoluble at the isoelectric point. The stalagmometric determinations were made with 2 per cent solutions of gelatin at 30° C., and with 0.45-0.49 per cent solutions of serum albumin at room temperature. Hydrogen ion concentration determinations were made by the indicator method using McIlvaine's standards made from 0.1 molar citric acid and 0.2 molar sodium phosphate.

Some of the experiments are as follows.

EXPERIMENT 1.

2 per cent solutions of granular gelatin. T = 30° C.

pH	No. of Drops (Stalagmometer)	pH	No. of Drops (Stalagmometer)
2.0	59½	5.0	59
2.6	57⅘	5 7	57⅕
3.2	57	6.5	55½
3.4	58	8.0	54
3.6	58½	9.0	53½
4.3	59½	9.4	53½
4.5	60½	10.0	55

EXPERIMENT 2.

2 per cent solutions of granular gelatin   T = 30° C.

pH	No. of Drops (Stalagmometer)	pH	No. of Drops (Stalagmometer)
2.0	60	6 0	58
2.4	59	6.4	57
3.0	58½	7.0	56
3.6	59½	8.0	54½
4.4	61½	8.4	55½
5.0	60	9 0	56½

EXPERIMENT 3.

0.49 per cent of ox serumalbumin. T = 10° C.

pH	No. of Drops (Stalagmometer)	pH	No. of Drops (Stalagmometer)
3.6	48¾	6.0	48⅕
3.8	47⅘	6.8	46⅘
4.0	47	7.4	47¾
4.6	47½	7.8	48
5.2	49⅘		

With the data from Experiments 1 and 2, the two curves of Figure 14 were constructed, and with those of Experiment 3, the curve of Figure 15. As is apparent in the case of gelatin, the surface tension of 2 per cent solutions

[64] J. Loeb, "Proteins and Theory of Colloidal Behavior," New York, 1922.

reaches a minimum at a pH of 4.4 to 4.5. Now this value of pH coincides approximately with that (pH = 4.7) found by Loeb and others for the iso-electric point of gelatin. On either side of this point in the acid or alkaline zones the surface tension increases, reaches a maximum, and then becomes lower.

With regard to serum albumin the surface tension is at its minimum at a pH of 4.6 to 5.2; a value which corresponds nearly to the isoelectric point
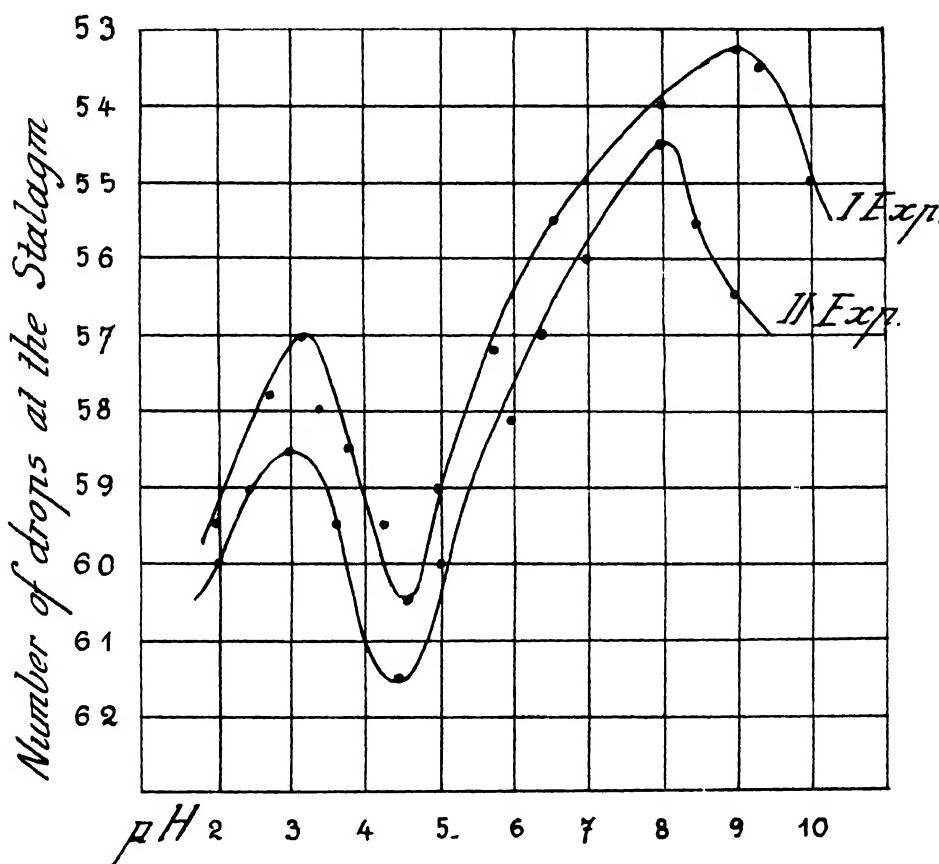
Fig. 14.—Surface tension changes in granular gelatin solutions.

of gelatin (pH = 4.7) and to that of crystallized ovalbumin (according to Sørensen, pH = 4.8). Similarly in this case there is noted first an increase and then a decrease in surface tension in the acid and alkaline zones; that is to say, on either side of the isoelectric point.

These recent researches completely confirm, then, the preceding results; wherefore one may conclude that the solutions of pure proteins have a minimum surface tension at the isoelectric point; and that as a consequence by means of determinations of surface tension as well as by means of deter-

minations of viscosity and of electrical cataphoresis, one can determine the isoelectric point of proteins which have the property of remaining in solution at that point (serum-albumin, hemoglobin, gelatin).

With regard to the interpretation of variations of surface tension that protein solutions undergo on varying the pH, we have already mentioned that given by Bottazzi and d'Agostino, and then accepted by Quagliariello; interpreting the increase in surface tension which one observes in passing the isoelectric point as due to ionization of the protein salt, and the succeeding diminution following the maximum in some degree to depression of dissociation
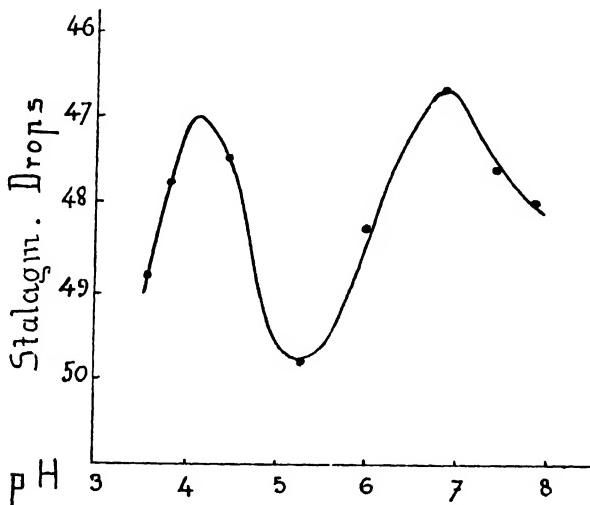
Fig. 15.—Surface tension changes in ox serumalbumin.

of the salt, that is, of the ionization of the protein. As a consequence we admit that proteins lower the surface tension of solvents to the maximum degree when they are found dissolved as undissociated molecules.

It is necessary, I said, to formulate an hypothesis that takes account of the lowering of the surface tension of protein solutions, after they have reached their maximum by the gradual increase of added acid and especially of alkali; and to explain the upward trend of the curves of Figure 8, beyond the points B,b,β (in the acid zone) and C,c,γ (in the alkaline zone) and the other corresponding curves obtained by Quagliariello and by me.

It has been pointed out that Traube noted that proteoses and peptones lower the surface tension of water considerably. Since at that time Traube denied the lowering action of protein substances it might be that the difference between the behavior of these and peptones was due to the fact that the protein "solutions" used by him were not solutions but suspensions.

It is significant that pure proteins undergo hydrolysis especially under the influence of strong bases, more than under the influence of acids, at room temperature and under very low concentrations of alkali.[65] Now if the first

[65] See Wo. Pauli, "Kolloidchemie der Eiweisskörper," Part I, p. 75, Dresden and Leipzig, 1920.

products of hydrolysis (also colloidal) have a lowering action greater than that of the proteins from which they were derived, the lowering in surface tension in the experiments described above might be due to the formation of hydrolysis products.

I do not deny that this may be in part due to repression of the electrolytic dissociation of the protein salt, as Pauli believes (*loc. cit.*, p. 80), in confirming the lowering observed by Wagner in which an excess of KOH to dialysed serum albumin finally caused precipitation. He states (p. 80): "On long standing there occur decomposition phenomena, and the coagulum diminishes and finally disappears."
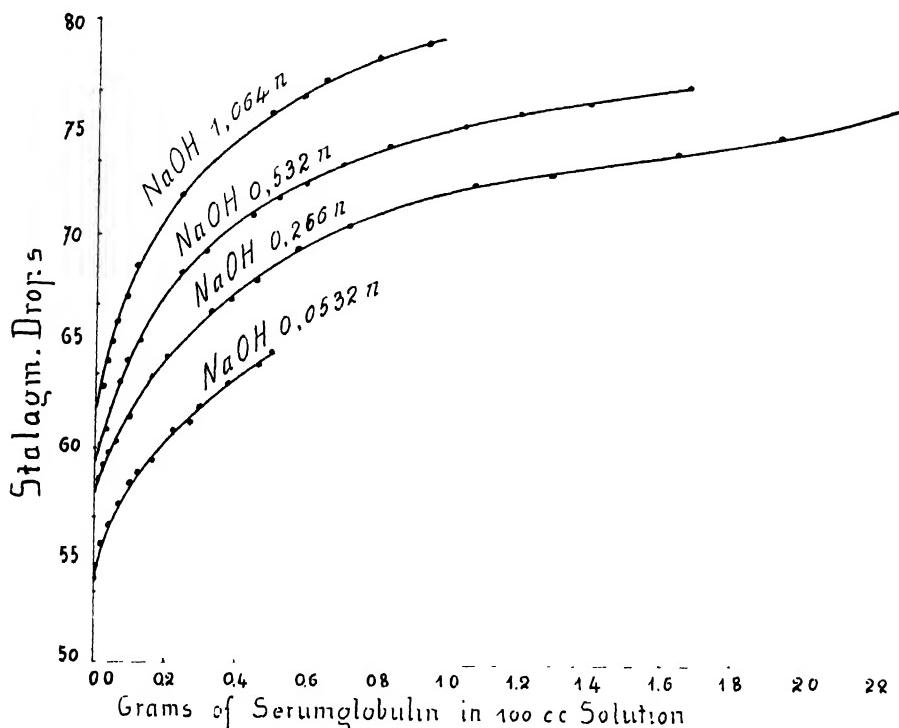
FIG. 16.—Graph of data in Table 16.

It is therefore possible that the lowering of surface tension may be due first to diminution of dissociation and then to hydrolysis.

The following experiments would seem to indicate this.

We have seen already that serum albumin dialysed for a long time and suspended in water does not lower the surface tension, but if dissolved with NaOH and shaken in contact with it for a long time in a thermostat at 28° C., the surface tension is considerably lowered, as the following experiment shows.[66]

[66] Fil. Bottazzi, *Rend. accad. Lincei* (5), 21 (2° sem.), 227 (1912).

Experiment: Distilled water .... 55 drops
Suspension of a great quantity of serumglobulin in water, after having allowed the excess to settle out............. 56½ "
The same suspension, after the addition of 5 drops of N NaOH solution, and after 24 hours in the thermostat at 28° C. (globulin not entirely dissolved)................. 63½ "
The same after the further addition of 10 drops of N NaOH, and after 24 hours in the thermostat (globulin nearly all dissolved) .... 71½ "
The same after adding 10 drops more of N NaOH (liquid yellowish-brown in color and a slight amount still undissolved) .... 76½ "

The solution thus obtained is very viscous, while the filtrate is limpid. If one dilutes this with distilled water the surface tension remains constant. When diluted progressively with N HCl, the surface tension is observed to rise a little (from 75¼ drops to 73¼ drops) only when a slight precipitate appears. Since the neutralization of the liquid does not have the effect of precipitating the dissolved protein entirely, and since the surface tension always remains low, one is led to conclude that NaOH has hydrolysed the serumglobulin (apparent also from the color change of the liquid) and that the lowering of surface tension is due to the hydrolysis products.

A similar experiment was made maintaining the concentration of NaOH constant, adding increasing quantities of serumglobulin to each solution, and

<div align="center">TABLE 16.</div>

Constant Concentration of NaOH=1.064 Mols per Liter		Constant Concentration of NaOH=0.532 Mols per Liter		Constant Concentration of NaOH=0.266 Mols per Liter		Constant Concentration of NaOH=0.0532 Mols per Liter	
Grams of Globulin in 100 Cc. of Solution	Number of Drops	Grams of Globulin in 100 Cc. of Solution	Number of Drops	Grams of Globulin in 100 Cc. of Solution	Number of Drops	Grams of Globulin in 100 Cc. of Solution	Number of Drops
0.000	55³/₁₀	0.0000	54⁹/₁₀	0.000	54½	0.000	53⅘
0.018	62⅜	0.0014	54⁷/₁₀	0.041	60²/₇	0.011	55
0.033	64⅛	0.0043	55⁴/₅	0.075	61⁰/₉	0.033	56⁵/₉
0.052	65⅛	0.0085	57½	0.128	63¼	0.061	57⁵/₉
0.066	66	0.0113	58³/₁₀	0.199	64¾	0.094	58⅝
0.093	67¼	0.0141	59	0.289	66½	0.117	59
0.116	68⁴/₇	0.0183	59¾	0.355	67	0.156	59⅝
0.232	71⁶/₇	0.0279	61	0.448	68	0.233	61
0.465	75⁰/₇	0.0470	62⅞	0.555	69½	0.292	61⅝
0.581	76⁵/₇	0.0650	64	0.729	70⁵/₇	0.333	62¼
0.664	77³/₇	0.1055	65	1.059	72³/₇	0.389	63
0.775	78²/₇	0.141	66	1.295	73	0.466	63¾
0.930	79	0.264	69	1.663	74½	....	....
....	....	0.325	69⅝	1.942	74⁶/₇	....	....
....	....	0.423	70⁵/₇	2.332	76²/₇	....	....
....	....	0.528	71⁵/₇	....	....	....	....
....	....	0.603	72¾	....	....	....	....
....	....	0.704	73²/₇	....	....	....	....
....	....	0.845	74⁴/₇	....	....	....	....
....	....	1.055	75³/₇	....	....	....	....
....	....	1.210	75⁶/₇	....	....	....	....
....	....	1.410	76⁵/₇	....	....	....	....
....	....	1.690	77²/₇	....	....	....	....

determining the number of drops from the stalagmometer after each addi-
tion. These results are summarized in Table 16 and plotted in the chart of
Figure 16.

It is apparent that the lowering of the surface tension was progressive,
and greater, the greater the quantity of NaOH added and the amount of
globulin present: in the first experiment, in fact, the number of drops indi-
cated by the stalagmometer increased from 62⅜ to 79.

SURFACE TENSION OF SUSPENSIONS AND SOLUTIONS OF SOAPS

The behavior of the higher fatty acids and their salts (soaps) fully con-
firm the facts described above regarding protein substances.

In 1910 Bottazzi and Victorow [67] observed: (1) that the purified hot
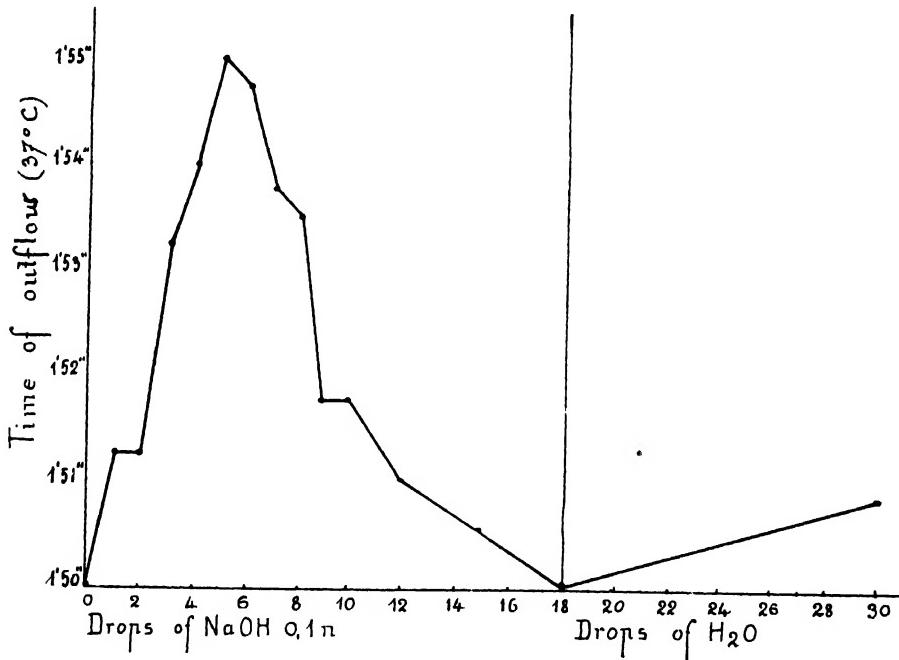
FIG. 17.—Surface tension changes in soap solution.

concentrated solutions of Castile soap ("Marsiglia soap") is very limpid and
optically void in the ultramicroscope; (2) that the same solution becomes
turbid and numerous ultramicroscopic particles appear when it is sufficiently
dialysed; (3) that the addition of NaOH clears the turbid liquid; (4) that
the suspended particles migrate toward the anode; (5) that the addition of
NaOH in increasing quantity caused the viscosity of the dialysed solution of
soap first to increase reaching a maximum, and then to diminish, finally attain-

[67] Fil. Bottazzi and C. Victorow, "Certain colloidal properties of soluble soaps," *Rend. accad. Lincei*
(5), 19 (1° sem.), 659 (1910).

ing its first value; [*] the clear solution became turbid, indicating the precipitation of the soap; (6) that the limpid solution of soap has a very low surface tension while the dialysed suspension had a surface tension but little lower than that of water; (7) that the addition of 0.1 *N* NaOH decreased the surface tension greatly, reaching a minimum, and then increased with continued addition of NaOH. These results are indicated in Figures 17 and 18.

In 1912 Bottazzi repeated experiments upon solutions and suspensions

FIG. 18.—Surface tension changes in soap solution.

of pure (Kahlbaum's) sodium and potassium oleate and stearate. With these experiments Bottazzi was able to demonstrate the following facts:

(1) The stearates [*] do not lower the surface tension of water, even though reduced to a state of fine division and visible as a skin upon the surface of the water. This is a confirmation of the fact that undissolved suspended material does not lower the surface tension of the dispersion medium, although it belongs to a group of substances (fatty acids and their salts) possessing in general great capillary activity.

(2) The 6 per cent limpid solution of pure sodium oleate is transformed

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[*] This seems to be an instance of the zone of maximum colloidality referred to by Alexander in Vol. I, Chapter 1. *J. A.*
[*] Zsigmondy showed that the protective action (gold number) of sodium stearate is much higher if its solution is made hot, which increases its degree of dispersion. *J. A.*

into a turbid milky hydrosol after prolonged hydrolysis. This hydrosol, which consists of a suspension of soap and of oleic acid in a dilute solution of sodium oleate, becomes clear and converted anew into a limpid solution upon the addition of NaOH in increasing amounts; while stalagmometric measurements show an increase in the number of drops from 75 to 106 indicating a great decrease in surface tension. The addition of NaOH in excess causes the solution to become turbid again, while the number of drops diminishes somewhat (*see* Fig. 19).

(3) If one adds NaOH in increasing amounts to a dialysed solution of 6 per cent sodium oleate to the point where the minimum surface tension is

Fig. 19.—Surface tension changes in sodium oleate solution.

reached and then at this point adds equivalent amounts of HCl, the surface tension is first lowered, then increases and when it reaches the maximum one observes a granular precipitate (*cf.* Fig. 20).

In this as in the preceding case the first lowering of the surface tension is evidently due to the fact that the NaOH dissolves the inactive aggregates suspended in the liquid transforming them into dissolved molecules of sodium oleate, which are capillarily active. A certain number of these undissociated molecules are always present in the system; they increase up to a certain point upon the addition of NaOH. The lowering of the surface tension is due to these undissociated molecules produced by the addition of NaOH. In fact the NaOH is not able of itself to increase the surface tension, while the oleic acid formed by the hydrolysis of the soap is inactive because it is insoluble. The excess of NaOH finally causes turbidity again and increases the surface tension a little because it precipitates the soap after having depressed the dissociation of the molecules to a maximum degree. Hydrochloric acid by neutralizing the NaOH nullifies these effects. In fact that portion of the curve to the right of 12 on the abscissa is symmetrical with that to the left.

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(4) The original 6 per cent solution of sodium oleate is never perfectly clear, because a certain number of molecules of the salt are hydrolytically dissociated and the fatty acid remains dispersed in the soap solution.

(5) If NaOH is added in increasing amounts to the solution, the latter is cleared and at a certain point becomes jelly like; when the amount of NaOH is very great the gel is broken up and the soap is precipitated as white clots.

(6) Finally the 6 per cent solution made appreciably alkaline remains clear and optically void in the ultramicroscope of Siedentopf and Zsigmondy, or at best shows a cone weakly and uniformly illuminated. Dilution or the addition of a few drops of acid causes the field to become strongly illu-
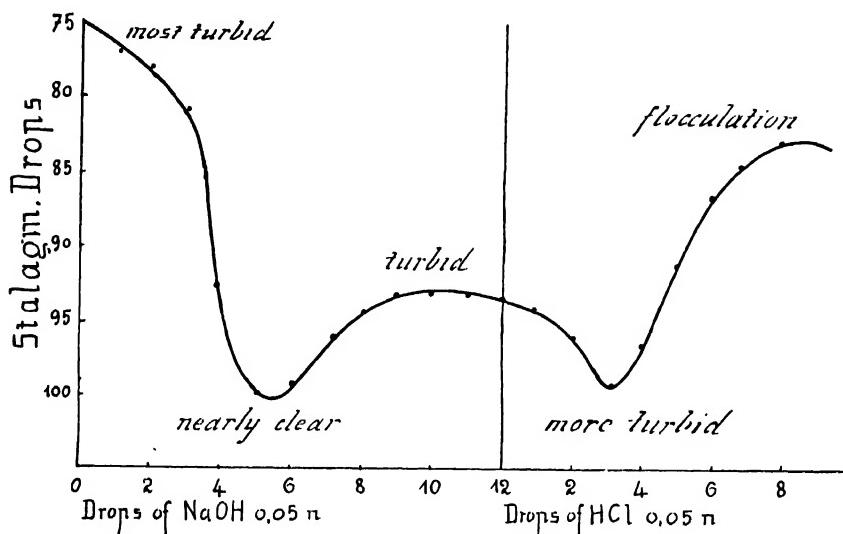
Fig. 20.—Surface tension changes in dialyzed sodium oleate solution.

minated and the particles to exhibit Brownian movement. Strong alkalinity of the solution is therefore necessary to prevent hydrolysis of rather concentrated solutions of sodium oleate.

In conclusion, the surface tension of solutions of sodium oleate increases with increase of hydrolytic dissociation of the salt and diminishes with diminution of the dissociation. In other words the undissociated molecules of oleate lower the surface tension of water, while the dissociation products are inactive, or, as with NaOH, increase it.

According to some authors, the surface activity of certain substances is concerned with their submicroscopic suspension rather than with their dispersion as undissociated molecules, as I have indicated above.

Windisch and Dietrich,[68] experimenting with the alkali salts of nonylic, caprylic, and undecylic acids found that: "On adding increasing amounts of acid to solutions of sodium nonylate, the surface tension of the solution exhibits a decrease exceeding that referable to the quantity of nonylate, and

[68] W. Windisch and W. Dietrich, "Über oberflächenaktiv und oberflächeninaktiv Modifikationen höherer Homologen der Fettsäurereihe und ihre Beziehung zu den Titrationen mit oberflächenaktiven Stoffen als Indicator," Kolloid Z, 26, 193 (1920).

with Na caprinate the decrease commences only after addition of the equivalent quantity of acid. This indicates that the fatty acid may also exist in a surface-active form, and becomes surface-active only after addition of acid. With undecylic acid it was proven that addition of acid led to formation of an active form, for a solution of pure undecylic acid showed 3 hours after making about the same surface tension as water; but after addition of 0.1 ccm. 0.1 N HCl powerful lowering of the tension supervened."

Now according to these authors, "The transition between the active and inactive forms of undecylic acid depends on the degree of dispersion. According to ultramicroscopic investigation, the inactive acid consists of amicrons, and the form activated by HCl of submicrons, while in the aged solutions (with the surface tension of water) the formation of large particles is proven by turbidity phenomena. . . .

"The molecularly dispersed phase of undecylic acid is therefore inactive, in the colloidal zone (submicrons) there occurs a maximum of surface activity, and in the zone of coarse suspensions the acid once more becomes inactive." [69] *

It is difficult to understand how matter in a state of submicroscopic dispersion is able to influence the surface tension of the dispersion medium; and how so much difference can exist between the effect of submicroscopic and microscopic particles of the same substance. It seems to me that the interpretation of this phenomenon should be different. When the solution is optically void, undecylic acid is found not in the molecular but in the ionic condition, and therefore does not lower the surface tension.

The addition of acid drives back the electrolytic dissociation; undissociated molecules then appear, and since the fatty acid is soluble in water, it lowers the surface tension of the solvent. Naturally the process of association with a substance slightly soluble in water does not stop at the formation of molecules, but continues with the formation of aggregates so that the liquid appears turbid to the naked eye. During the process of association, therefore, molecules should appear even though their isolated existence is transitory.

From what one reads in "Referat" [70] it is apparent that in order to lower the surface tension of solutions of the salts of these fatty acids, the authors had to add "an amount of acid in excess of the nonylate equivalent." This shows that the salt was electrolytically dissociated to some extent.

The work of Windisch and Dietrich was apparently inspired by some preceding experiments of Traube and Onodera [71] on the toxicity of alkaloids in relation to the surface tension of their solutions and with the size of particle into which they are dispersed. These authors observe that when an alkali is added to a solution of a salt of an alkaloid which in general lowers the surface tension of water "there often follows a considerable lowering of the surface tension, varying with the kind and quantity of the alkaloid liberated. This diminution of surface tension indicates an increase in poisonousness of the solutions. . . ."

But the free alkaloids are very unstable in their solutions; they flocculate, and with increase in size of the granules and of the surface tension of their

* This seems to be another instance of the zone of maximum colloidality. *J. A.*
[69] From *Ber. ges. Physiol.*, 2, 196 (1920).
[70] I have not been able to consult the original article.
[71] J. Traube and N. Onodera, "Ueber den Kolloidzustand von Alkaloiden. Beziehungen zwischen Oberflächenspannung, Teilchengrosse und Giftigkeit," *Intern. Z. physik. Chem. Biol.* 1, 35 (1914).

solutions their toxicity diminishes, because evidently the alkaloids no longer exist in solution, but pass into a state of suspension of increasing particle size.

These authors do not state clearly whether they consider undissociated molecules of the alkaloids as responsible for the decrease in surface tension, or as determining the toxicity. They tend, it seems, to refer the toxicity and the surface activity of these solutions to the degree of particle size rather than the molecular dispersion of the alkaloids. "The solutions of free alkaloids are not so very stable. Particle size and surface tension increase, and poisonousness correspondingly decreases. If some alkali be added to the more or less detoxicated solution, particle size and surface tension decrease and poisonousness correspondingly increases." Now the alkali added increased the degree of dispersion of the alkaloid, probably through adsorption of OH⁻ precisely as with metallic colloids, and thereby made possible the existence of a small fraction of the alkaloid as undissociated molecules. But evidently the authors do not attribute the toxicity and lowering action to this.

Later Traube was converted to the explanation formulated by me many years ago; that is, that the lowering action is due to undissociated molecules, not ions or submicrons. In fact in 1923 Traube [72] writes (p. 22); "Alkaloid ions are molecularly dispersed in water, and because of their ionic charge have a high solution-pressure, because of which they are not surface-active and incapable of aggregation into submicrons.

"On the other hand, alkaloid particles deprived of their ionic charge have mostly a very slight solution-pressure, and are surface-active and capable of combining into submicrons.

"In such freshly prepared alkaloid solutions, prepared either directly or by addition of alkali to solutions of alkaloid salts, in addition to numerous actively moving ultramicrons, there are presumably also numerous molecular dispersed particles. It is these latter which materially influence the surface activity.

"As the solutions age, the actively moving submicrons aggregate to larger immobile submicrons, the surface-active solutions becoming meanwhile more and more surface active. See especially the experiments on atropine solutions. Traube and Onodera, *loc. cit.* p. 37. Furthermore, if increasing quantities of alkali are added to the solution of an alkaloid salt, e.g. quinine chlorhydrate, the surface-activity increases just as long as the solutions remain clear. However, as soon as stronger turbidities appear, that is, larger submicrons form, the surface tension increases, that is, the surface-activity decreases. It is, of course, comprehensible that when in molecularly dispersed state, the particles which form a submicron lower the surface tension much more powerfully than when in submicronic aggregation."

Apparently this became "comprehensible" to Traube, after he had information of Bottazzi's researches, probably through Höber,[73] and of his interpretation of them.

But the impression that Traube is entirely free of the error of supposing a submicronic suspension capable of lowering the surface tension, is annulled when he writes in the same work as follows: "Fatty acids and their

[72] J. Traube, Hauptdruck, Oberflachenaktivitat und die Tendenz zur Submikronenbildung, *Kolloid Z.*, 32, 22 (1923).
[73] In fact R. Höber writes ("Physikalische Chemie der Zelle und Gewebe," 5th ed., Part I, pp. 177-178, Leipzig, 1922): "Nach Bottazzi hat eine Albuminlosung bei derjenigen Reaktion, die dem isoelektrischen Punkt des Eiweisses entspricht, ein minimum der Oberflachenspannung; die Neutralteilchen haben also offenbar eine grossere Oberflachenaktivitat als die Eiweissionen." Bottazzi had demonstrated in 1909 that proteins as submicrons do not exercise a lowering action.

water-soluble salts behave analogously to the alkaloids and alkaloid salts.
Fatty acid ions in the aqueous solutions of alkali salts of the fatty acids generally go into molecularly dispersed solution, their solution pressure is high, the solutions are surface-active, submicrons are not present.

"Free fatty acids (apart from formic acid) have a slight solution pressure; they are surface-active and from butyric acid on are capable of aggregating into submicrons. Here, too, as with the alkaloids, the solution pressure decreases with increasing molecular weight, whereas the surface-activity and the ability to form submicrons increase."

He then adds that . . . "on the other hand, butyric acid solutions on the contrary contain numerous submicrons, and from the investigations of Windisch and Dietrich [*Kolloid Z.*, **26**, 193 (1920)] as well as of Traube and Klein [*Kolloid Z.*, **29**, 242 (1921)], it appears that the higher fatty acids, nonylic, caprinic, and undecylic acids, are not only highly colloidal, but may, according to the size of their submicrons, exist in a surface-active as well as a surface-inactive form in the solution. Just as with the alkaloids large submicrons may be broken up into smaller ones by addition of alkali, thus becoming surface-inactive, so with the higher fatty acids referred to, addition of HCl produces the same result."

Wherefore he considers that a greater degree of dispersion of the three fatty acids and of the alkaloids still remaining within the confines of colloidal dispersion or submicrons would be sufficient to cause the substance to pass from "surface-inactive" to "surface-active," quite forgetting that he had written previously "that in molecular dispersion, the submicron-forming particles (that is molecules) depress the surface tension much more powerfully than in submicronic aggregation."

He concludes: "For many aqueous solutions of surface-active materials the following rule holds: The lower the solution pressure, the higher the surface-activity in the sense of Gibbs, and the tendency to form submicrons."

Very well! Certain substances which lower the surface tension tend to form submicrons because of their feeble electrical dissociation and hydration capacity. There is a great tendency toward association—aggregation into submicrons, but the lowering of surface tension is due to the dissolved molecules, not to the suspended submicrons.

The recent researches of Lascaray[74] on the surface tension of soaps are most interesting. In the first place there is found in the work cited (page 77) a confirmation of what I have already observed, namely that the stearates and palmitates are but little endowed with "surface activity" (evidently because they are almost insoluble in water): "Up to these members (Na-palmitate and Na-stearate) of the series, the surface activity of soap solutions is larger the larger the molecular weight of the fatty acid. Therefore the soaps form a regular series in increasing surface activity, as is the case with the fatty acids themselves. Sodium myristate, however, reaches a maximum of surface activity. . . . With higher molecular weight, the surface activity is considerably smaller." *

Moreover he has confirmed (pages 77-78) the fact that solutions of sodium oleate gelatinize at a certain concentration, but I have established the relation between gelatinization and the concentration of alkali progressively added. The author discusses those parts of an aqueous solution of soap which tend

74 L. Lascaray, "Ueber die Oberflächenspannung von Seifenlösungen," *Kolloid Z.*, **34**, 73 (1924).
* See also Chapter 1, Vol. I, of this series. *J. A*

to lower the surface tension of the solvent and arrives at the following conclusions: "That molecularly dissolved fatty acid (that is, when the fatty acid is soluble in water) lowers it, and Na-ions raise it, is beyond question. Fatty acid ions have very little influence on the surface tension of water, and may therefore be neglected. . . ."

"No conclusion can be drawn as to the influence of soap molecules on the surface tension of water, for it is not possible to make an aqueous solution of a soap which is not dissociated into ions."

However, the following conclusion is admissible: "Soap molecules occupy an intermediate position between NaOH (which increases the surface tension) and fatty acids (which lower it). And also this, ". . . soap molecules are the more surface-active, the higher we go in the acetic acid series"—probably because in the same sense there is a diminution of their affinity for the solvent.

Lascaray says quite justly "that the colloid portion (that is, that part which exists as dispersion submicrons) of a soap solution influences the surface tension but little (he could have said, "not at all"). . . . The influence of the colloids of a soap solution is confined mainly to the adsorption of ions and molecules, which otherwise might have affected the surface tension."

Finally, by increasing the concentration of soap solution, the fatty acid of which is but slightly soluble, the acid which separates by **hydrolysis** emulsifies in the soap solution; one can always admit in this case "that in the emulsion of a fatty acid, it is essentially the molecularly dissolved portion of the fatty acid which influences the surface tension."

The lowering of surface tension produced by oleate soaps where the fatty acid (oleic) is insoluble, is essentially due to undissociated soluble molecules of the soaps. Hydrolysis does not increase the surface tension of the solution. The stearate does not lower it to any degree, because it is practically insoluble. It is quite apparent that the results obtained by Lascaray are in perfect accord with those of Bottazzi.[75]

General Conclusions

It seems to me that from what I have explained above one can draw the following conclusions regarding the influence that certain protein substances, alkaloids, soaps, etc., exert upon the surface tension of water. These substances are capillarily active only when existing in a state of true solution, while they are inactive when found dispersed as submicrons (or colloidal). Hydrolysis (submicronic dispersion) causes a surface tension somewhat lower than that of the pure dispersion medium, but this probably depends upon the fact that the system contains a part of the substance, even though little, dispersed as dissolved molecules.

Of the soluble substances, the undissociated molecules—not the ions—are responsible for lowering the surface tension. The minimum lowering of surface tension of a protein solution always corresponds to the isoelectric point of the dissolved protein. That is to say, we have a means of deter-

[75] The accord is so perfect that the shape of my curve expressing variations of surface tension caused by the addition of increasing amounts of NaOH to a 0.6 (about 0.197N) dialysed sodium oleate solution is similar to that of the curve constructed from data of Table XIV of Lascaray concerning variations of surface tension of solutions of sodium oleate of increasing concentration. And the similarity is not to be wondered at if one considers that the addition of NaOH to a solution of dialysed sodium oleate (in which the greater part of the soap is precipitated) is equivalent to increasing the concentration of the soap solution. This similarity is borne out in curves representing the behavior of sodium laurate.

mining the isoelectric point of a soluble protein by measuring the surface tension of its solutions.

Undissociated molecules of a protein, of an alkaloid, of a soap, etc., are generally less hydrated than their ions. They have a greater tendency to aggregate into submicrons. When one reduces the electrolytic dissociation of a salt of a protein, of an alkaloid, or of a fatty acid, etc., the process of association does not stop at the formation of undissociated molecules, but proceeds further to the formation of molecular aggregates, that is, of submicrons and of microns. Simultaneously the surface tension of the liquid is lowered, but the lowering of the surface tension is not due to their formation. It is due instead to the formation of undissociated, free molecules of protein, of alkaloid, of fatty acid, etc., which remain invisible in the ultramicroscope.

In conclusion, substances which lower surface tension exert their depressing effect when they exist in the state of dissolved molecules, not in that of ions or that of molecular aggregates.

Nephelometry

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In general, it may be said that the progress of chemistry has been dependent upon the development and refinement of quantitative methods of analysis. Indeed, the introduction of the analytical balance marked a new era in the science of chemistry, for no other single instrument has contributed more to the development of the science. From time to time, other instruments have been devised for the quantitative study of the chemical elements, their compounds, reactions, physical constants, and even their internal structure. Among these instruments we may mention: the graduated tube, burette, pipette, graduated flask, electrolytic apparatus, electrometric apparatus, colorimeter, nephelometer, apparatus for X-ray analysis, and the mass-spectra apparatus. The last two named instruments are, indeed, quite recent, but they are not employed in the ordinary quantitative analysis. Although the first nephelometer was introduced over thirty years ago (1894), its general use in quantitative analysis did not begin until eighteen years later (1912), when Kober showed that nephelometry could be made sufficiently accurate for most analytical purposes. Within a few years several types of nephelometers were on the market at a reasonable price and these now take their place among our instruments of highest precision and accuracy. An idea of their extreme sensitiveness is gained from Kober and Egerers'[1] nephelometric reagent for phosphorus, which will detect 1.0 part in 333 million parts of water; Graves' nephelometric reagent for ammonia,[2] which will detect 1.0 part in 160 million parts of water; and Marriott's[3] nephelometric reagent for acetone, which will detect 1.0 part in 100 million parts of water. Many substances can be quantitatively determined nephelometrically when their concentration is of the order of a tenth to one part per million parts of water. Indeed, the nephelometer is limited to the measurement of substances in *low concentration*, usually not stronger than 100 milligrams per liter. To apply the method to larger amounts of substance it is only necessary to dilute suitably. There are certain other limitations, but these will be discussed subsequently.

Quantitative analysis until recently has depended chiefly upon the formation of practically insoluble precipitates which can be separated from the surrounding medium either by sedimentation or by filtration. The precipitate is then washed, dried, and weighed. This method, as all analysts know, is long and often quite tedious, and requires considerable practice, patience and skill. The requirements of the precipitate are many. It must contain all of the constituents, i.e., it must be insoluble or practically insoluble; it must be pure; and must have a definite and known composition. Moreover, the precipitate must be in a form permitting reasonably rapid filtration and

[1] *J. Am. Chem. Soc.*, **37**, 2375 (1915).
[2] *Ibid.*, **37**, 1171 (1915).
[3] *J. Biol. Chem.*, **16**, 289 (1913).

highly accurate weighing. It must not change in composition upon drying or being ignited, or if it does, it must change to a substance of definite and known composition and be in a condition suitable for weighing.

Fortunately, the chemist is not dependent upon gravimetric methods entirely. Frequently a volumetric or a colorimetric method may be used. Often these are just as accurate and much shorter. However, most determinations depend upon gravimetric methods. Obviously, a direct method of estimating the mass or weight of precipitate in solution (or rather, in suspension), i.e., without filtering, washing, drying, and weighing, would be highly desirable. Such a method we now have in *nephelometry*. Both the name and the original apparatus are due to T. W. Richards.[4] The word *nephelometry* is derived from the Greek, νεφέλη, meaning a *cloud*. The method is based upon the measurement of the brightness of the light reflected by a cloud—that is, by finely divided particles in suspension—very much as in an ultramicroscope. In other words, nephelometry deals with two phase systems. One phase consists of finely divided solid or liquid particles; the other consists of a liquid. The former is sometimes referred to as the discontinuous phase, the latter, as the continuous phase. Perhaps in colloid chemistry they are more frequently called the dispersed phase and dispersing phase or dispersion medium, respectively. Since the indexes of refraction of the two phases are (usually) different, these suspensions appear turbid. The intensity of the light reflected by these turbid solutions is a function of the amount of suspended particles, other conditions being kept constant.

History

Although Gay-Lussac invented the volumetric method of estimating silver, apparently he did not employ any special apparatus for judging the amount of opalescence. Mulder,[5] however, roughly compared opalescent silver chloride suspensions obtained in the supernatant liquor, or the filtrates, in his atomic weight determinations. This he accomplished by keeping the flasks, containing the silver chloride, in metal boxes blackened inside, and from time to time raising them before the diffused light of a window. Thus he roughly judged the amount of opalescence. Sometimes he decanted the supernatant liquors into small tubes and the amounts of opalescent precipitate were gauged roughly with the eye. His comparisons were wholly a matter of judgment; he used no measuring device. His experiments, however, mark the first step in the development of nephelometry. The second step was made by Stas,[6] in 1894, who used a series of tubes with perfectly plain bottoms. Four tubes, about 4 cm. in diameter, were supported adjacent to each other upon a shelf, over holes of the same diameter as the tubes, and beneath which was an illuminated scale. Everything above the shelf was kept in darkness. When the marks on the scale viewed through two heights of opalescent solution appeared the same, Stas assumed there was an equal weight of suspension in the two tubes. The great disadvantage of this instrument is that the light is transmitted through the precipitate. If instead, the reflected light is observed, when a powerful beam is allowed to fall upon the precipitate obliquely, the delicacy of the estimation is greatly increased. It was upon

[4] *Proc. Am. Acad., Arts Sci* **30**, 385 (1894)
[5] "Die Silber Probiermethode," Trans., Grimm, Leipzig, 1859, p. 23.
[6] *Œuvres*, 1, 155 (1894).

the latter fact that Richards,[7] in 1894, devised the instrument which he named the "nephelometer."

EARLY TYPES OF NEPHELOMETERS

First Nephelometer (Richards, 1894). The first real nephelometer, as above mentioned, was made by Richards to compare opalescent precipitates in connection with his atomic weight work on strontium, and was described in a paper on the latter subject. A diagram of the instrument is shown in
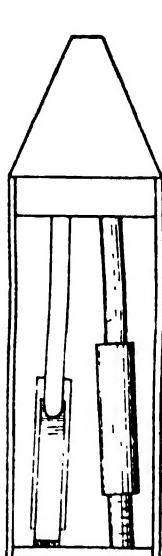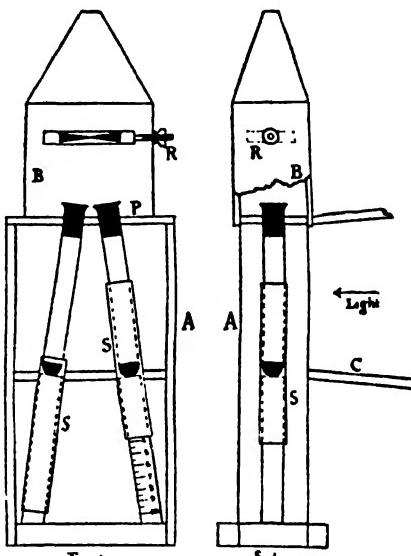
FIG. 1.   FIG. 2.

[from *J. Ind Eng. Chem.*, **7**, 844 (1915)]

FIG. 1.—First Nephelometer. Made by Richards in 1894. Substance contained in two test-tubes; observed from above. Sliding jackets (graduated) were used to match the light reflected.

FIG. 2.—Second Nephelometer. Made by Richards and Wells in 1904. The sensitiveness of the instrument was increased by painting the ends of the tubes and inserting two prisms.

Figure 1. Two test-tubes were arranged almost vertically, but slightly inclined toward one another so that the eye could look into both. Around the test-tubes were two opaque sliding jackets. When the slides were adjusted so that the incident light was cut off in such measure as to give equal opalescence in the two tubes, the precipitate was taken as inversely proportional to the lengths exposed to the light. This relation does not hold accurately for dense precipitates, since the nearer portion partly hides the more distant ones; but for slight opalescence the error is not great, especially when the lengths are not very different. With care, considerable accuracy could be attained, but to secure good results, long practice was necessary.

[7] *Proc. Am. Acad. Arts Sci.*, **30**, 385 (1894).

Second Nephelometer (Richards and Wells, 1904).[8] In order to reduce the uncertainty of comparison due to the inevitable distance between the centers of the two test-tubes, Richards and Wells modified the first nephelometer and made a number of improvements. The instrument is shown in Figure 2. It consists essentially of three separate parts: the main frame *A*, which holds the tubes in position, a movable top *B*, containing adjustable prisms, and a large box *C*, in which is a source of light.

The tubes destined to hold the solutions under examination were testtubes of clear glass free from striations, and containing each 0.032 liters, painted outside around the top and bottom with black asphalt paint. These opaque bands form the most convenient method of obliterating reflections from the meniscus and the curved bottom of the test-tube. The space between the lower edge of the upper band and the bottom of the tube was exactly the same in each case. The lower edge of the upper band projected below the wooden support, *P*, in order to provide a sharp line of demarcation between light and darkness, as well as to allow the tubes to be shut into complete darkness by the sliding jackets, *S*, *S*. The tubes rested upon equal wooden pillars (which guided the sliding jackets) and projected well above the support, *P*, in order to preserve their cleanliness The sliding jackets were also of glass, thickly painted. They moved up and down over the test-tubes, shutting off as much light as was wished and being held in any desired place by a brass spring. When raised, they disclosed below two scales which indicated exactly the lengths of the tubes above exposed to the light.

Upon the frame rested the small box *B*, which shut out all light, and yet was easily removable. In this box was a small frame which could be adjusted to position by means of the set screw *R*. This frame contained two 15° prisms with their thin edges together, the edges having been ground slightly by an optician until they fitted closely. The effect of the prisms when looking downward through them is to bring into view, side by side, semicircular images of about half of each tube, so that the two halves appear scarcely larger than a single tube, the dividing line being barely visible. The appearance of the image resembles the field of a half-shadow polarimeter. The success of the instrument lies in this arrangement of the prisms. Different observers can judge the equality of two tubes to within a few per cent, an accuracy impossible with the older nephelometer where the mind had to estimate the small differences at a distance; under favorable conditions successive readings of the scale by one observer will not vary so much as a millimeter.

Third Nephelometer (Kober, 1912). Up to this time Richards' instrument and previous apparatus were designed especially to estimate small quantities of substances in filtrates, in order to obtain a correction for certain atomic weight determinations. For this purpose it served excellently, but it had one disadvantage as an instrument for general analytical work, namely, best results were obtained only by taking the average of a large number of readings.

As a matter of fact Richards[*] believed nephelometry was not suitable

[8] Richards and Wells, *Am. Chem. J.*, 31, 235 (1904).
[*] "This instrument is not intended for determining large amounts of substance which deposit easily from solution; ordinary quantitative methods serve much better in such cases. Its great usefulness appears when one is required to determine minute traces of precipitates which obstinately refuse to settle, or to be caught by an ordinary filter or Gooch crucible. It may be used not only with silver chloride, but also in many other cases in which a fine divided precipitate reflects light; and it provides an unusually sensitive means of detecting very faint cloudiness in a liquid." Richards, Orig Com. 8th Intern. Congr. Appl. Chem., 1, 426 (1912).

for general analytical work, owing to its inaccuracy. Richards' erroneous opinion was based upon his experience with silver chloride suspensions, which as Kober[9] and Kleinmann[10] later showed are, unlike most suspensions, very difficult to obtain in a condition suitable for nephelometric measurements. More recently, however, Lamb, Carleton and Meldrum[11] developed a special technique whic produces silver chloride suspensions suitable for nephelometric work.

Kober considered that an instrument similar to Richards, but of greater accuracy, and one which would yield reliable results with a few readings, com-
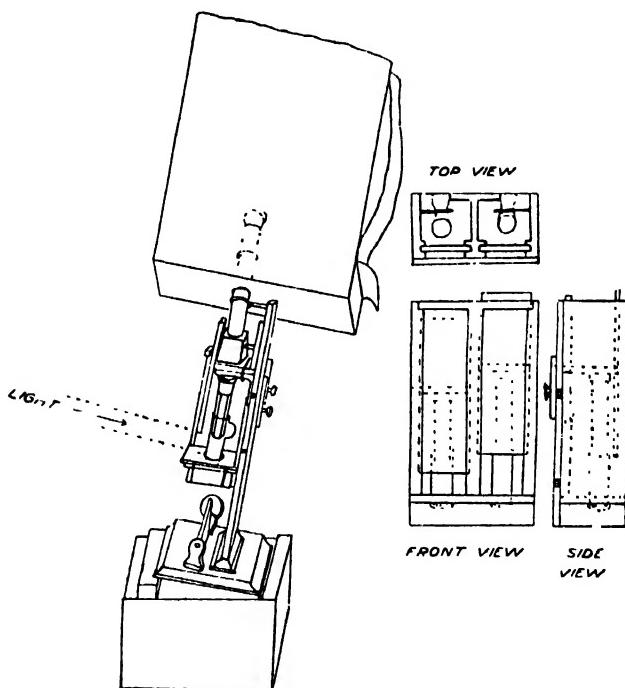
FIG. 3.—Third Nephelometer. Made by Kober in 1912, from a Duboscq colorimeter with wooden attachments. This instrument eliminated errors due to the meniscus and produced a much better optical equipment.

parable to those obtained with a Duboscq colorimeter, would greatly enhance the value of the nephelometer for the chemical analyst; and so in 1912 he build a nephelometer from a Duboscq colorimeter with wooden attachments made from small packing box boards (Fig. 3). The only changes required were a coat of black asphalt paint on the plungers, a special nephelometric tube and a receptacle for the same. This instrument eliminated errors due to the meniscus, and had a much better optical equipment.

[9] *J. Ind. Eng. Chem.*, **10**, 564 (1918).
[10] Inaugural Dissertation, Berlin, **1919**, p. 119; *Biochem Z*, **99**, 115 (1919)
[11] *J. Am. Chem. Soc.*, **42**, 251 (1920).

Fourth Nephelometer (Kober, 1913).[12] The third nephelometer gave good results, but the wooden parts soon began to warp due to moisture, so in the next instrument the wooden parts were replaced by metal. In other respects this nephelometer is similar to the third. In addition to increased accuracy over the Richards instrument, it has the greater advantage of being still usable as a colorimeter. The change from colorimeter to nephelometer and *vice versa,* can be made in a few minutes. Although the instrument gives very good results, it has several disadvantages: (1) it is limited to about 40 mm. height of liquid, (2) the plungers require repeated painting on account of the solvent action of some liquids and the peeling off of paint through use, (3) dissolved or minute flakes of paint might introduce an unexpected error, and (4) its cost is relatively high.

Kober Improved Nephelometer-Colorimeter. Kober's 1917 instrument was a decided advance in the construction of nephelometers. A detailed de-
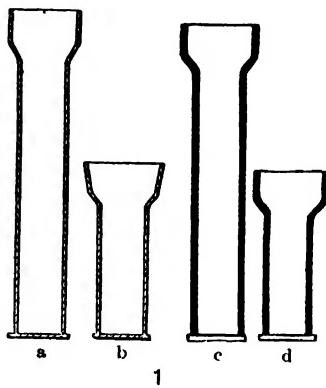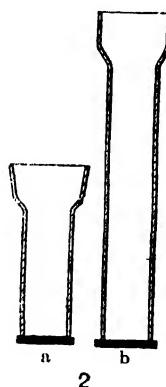
Fig. 4.   Fig. 5.

FIG. 4.—Colorimetric cups. (*a, b*) Transparent and optically clear bottoms fused to the sides; (*c, d*) black glass sides which eliminate the colorimetric light shield.

FIG. 5.—Nephelometric cups. (*a, b*) Black glass bottoms fused to transparent sides. Diameter of cups just ½ mm. larger than that of the plungers.

scription of the instrument would be out of place in this brief treatment of nephelometry, but the improvements over earlier types may be summed up as follows: (1) the use of a screw arrangement with adjustable verniers, also double milled head; (2) the screw arrangement being located in the back of the instrument, is away from possible contact with corroding liquids overflowing from the cups; (3) removable scale, making it possible to repair or to replace by a new one; (4) no open spaces are to be found between the top of the eye-piece and the liquids in the cups so that dust is excluded; (5) the scale and instrument are so constructed that any height of liquid up to 110 mm. can be measured; (6) the colorimeter cups, (Fig. 4) like the nephelome-

[12] During the summer of 1913, W. R. Bloor working in the chemical laboratory of Queen's University, Kingston, Canada, converted a Schreiner colorimeter into a nephelometer and used it in working out a method for the determination of fat in milk. This method was reported "by title" at the Rochester meeting of the American Chemical Society in the fall of 1913. Later in St. Louis, Mo., he continued his work on the nephelometric method for fat in milk and finally reported his results in the *J. Am. Chem. Soc.*, **36**, 1300 (1914).

ter cups (Fig. 5), with which they are interchangeable, are fused instead of cemented and are therefore of one piece of glass, usable for all solvents; (7) black glass plungers (Fig. 6), with fused-in, optically clear bottoms, not cemented, makes its use as a colorimeter and nephelometer a matter of equal ease and accuracy, entirely eliminating the use of asphaltum paint for this purpose; (8) a new design of a field (Fig. 6), similar to that of Lummer-Brodhun, a square within a circle, makes its use, when correctly made and adjusted, more sensitive and much more comfortable for the eye.

After several years of use, the 1917 model of the Kober instrument was further improved. In the 1921 instrument (1) the milled heads, formerly at the top of the instrument, are placed at the bottom, which allows the hands to rest on the table or other support and the adjustments to be made with the fingers. (2) An auxiliary scale is provided at the top of the instrument consisting of: two scales engraved upon the side away from the operator, fastened to the movable stages, so that when the stage is being moved up or down, the scales move with it; a stationary vernier, protruding beyond the top plate, also engraved upon the side away from the operator, fastened at the top of the instrument. A mirror facing the operator at an angle of 45°, is placed in front of the protruding scale and vernier, so that an image of the two is reflected vertically. A magnifying glass of the same focal distance as the telescope, serving as a second eyepiece, has been placed close beside the regular eyepiece, directly above the mirror, showing the image of the scale enlarged in good light.* The setting of the zero point is easily and accurately accomplished with a micrometer arrangement, involving a milled head working against a spring. This convenient method of zero point adjustment, together with the very simple method of using the instrument, the method of Lamb, Carleton, and Meldrum, where the height of

FIG. 6.—Prism arrangement and plungers. Black glass plungers with optically clear glass ends, fused together. Prism arrangement, which gives a field similar to that of Lummer-Brodhun.

the standard solution is kept constant, makes the operation of the instrument and the calculation of results extremely simple and easy without, however, sacrificing accuracy or deviating from the fundamental basis of either colorimetry or nephelometry.

In Figure 7 is shown the instrument attached to a lamp house.

Kleinmann Macronephelometer.[13] [14] The nephelometer is shown in Figure 8 (an objective front view) and Figure 9 (a diagrammatic sketch of the design in side elevation).

Figures 8 and 9 show the two test tubes a and a_1 into which the standard solution and the solution to be tested are filled. They hold about 12 cc. each. These test tubes are carried in metal casings b and b_1, in which they fit easily,

so that they can be moved up and down without difficulty.   The casings are fitted on spring bases adapted to slide in a suitable frame.

A beam of light is thrown on the test tubes by a lamp placed in front of the instrument and the Tyndall-cones thus produced are observed and gauged in a line perpendicular to the axis of the beam.

For this purpose the diffracted light is made to pass first through two solid glass cylinders *c and* c_1, identical in shape and size and cut out of adjacent
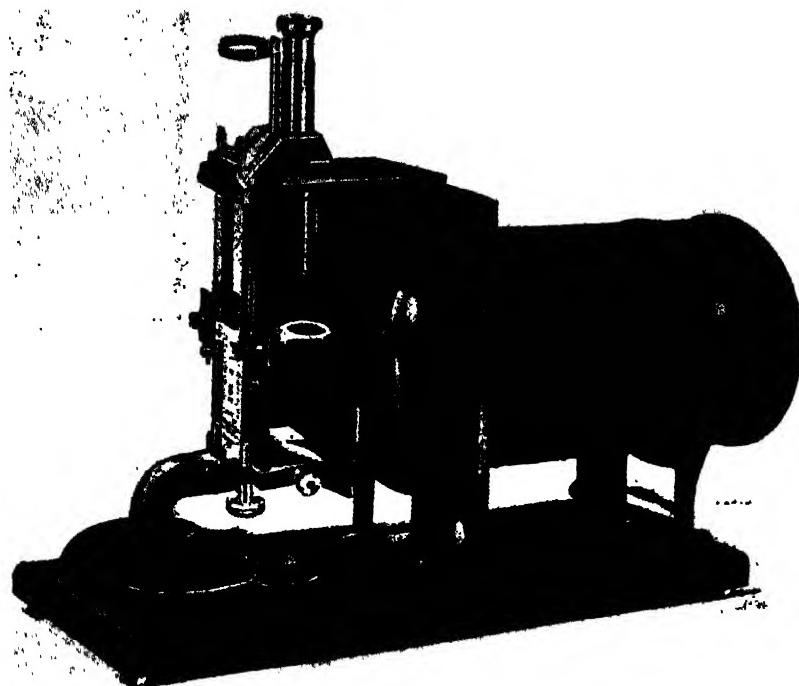
[from *J. Lab Clin Med*, **12**, 629 (1927)]

FIG. 7.—Kober's Improved Nephelometer-Colorimeter.

parts of the same block of glass, in order to render their action on the light absolutely symmetrical.   To eliminate the error which may be caused by observing the surface of the liquid, the lower parts of the cylinders are immersed in it.   By a suitable arrangement of diaphragms the cylinders receive light only from the central part of the Tyndall-cones.

The section of the tubes exposed to light, and therefore also the diameter of the Tyndall-cones, can be varied at will by varying the height of the windows f and f_1 through which the light reaches the turbid solutions.   These windows are about 4.5 cm. high and about 2 cm. wide.   The bottom part of the window openings is closed by a movable metal plate with a sharp edge, fitted on the interior surface of the wall of the instrument so that the shadow limiting the illuminated section is very sharply defined.   These metal shutters can be displaced by means of rack and pinion and the displacement read

by means of verniers. The height of the windows can be varied independently for each Tyndall-cone, by means of the corresponding milled screw heads t and t_1, from complete closure to the full height of 4.5 cm.

The verniers may be conveniently read from the back, the observer's side, of the instrument in the prisms L and L_1, that receive light from the observation lamp and are adapted to be moved laterally to bring the scale into focus.

The scale is divided into millimeters and reads from zero (closed window) to 45 (window opened full). By means of the verniers the scale allows
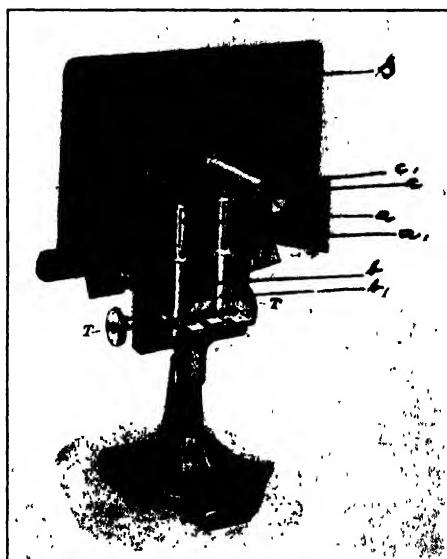
Fig. 8.—The Kleinmann Nephelometer.
[from *J. Lab. Clin. Med.*, 12, 629 (1927)]

reading of 0.1 mm. The observer is screened from the light of the lamp by a removable screen S. In order to exclude the light reflected from surrounding objects the turbid solutions are enclosed in a box of blackened sheet-metal, not shown in the drawing, which is permanently fitted on the instrument and can be easily opened and closed.

A frosted Osram lamp of 100 candlepower is preferably employed as source of light. It should be installed at a distance of 75 cm. in front of the instrument in a line with its optical axis and on a level with the windows.

Kleinmann's Micronephelometer.[15] In cooperation with the firm of Schmidt and Haensch, Kleinmann constructed a supplementary fitting to be used in the above-described nephelometer in place of the test tubes containing the solutions. By means of this modification the instrument may be used both as macronephelometer (taking 12 cc. of solution) and as micronephelometer, taking smaller quantities, down to 2.6 and 1.5 cc.

For this purpose the test tubes R_1 and R_2 (Fig. 10), of smaller diameter

[15] Kleinmann, *Biochem. Z.*, 137, 148 (1923). The present description of the instrument is from Kleinmann, *J. Lab. Clin. Med.*, 12, 629 (1927). Supplied by the firm of Schmidt and Haensch, Berlin, Prinzessinnenstr 16, or by Akatos, Inc, 114 118 Liberty Street, New York City.

and shorter than the tubes ordinarily used, are provided, R_1 holding 2.6 cc. of solution, R_2 1.5 cc. The latter is fitted with a glass stopper. Two glass cylinders for submersion in the solution are further provided, of smaller
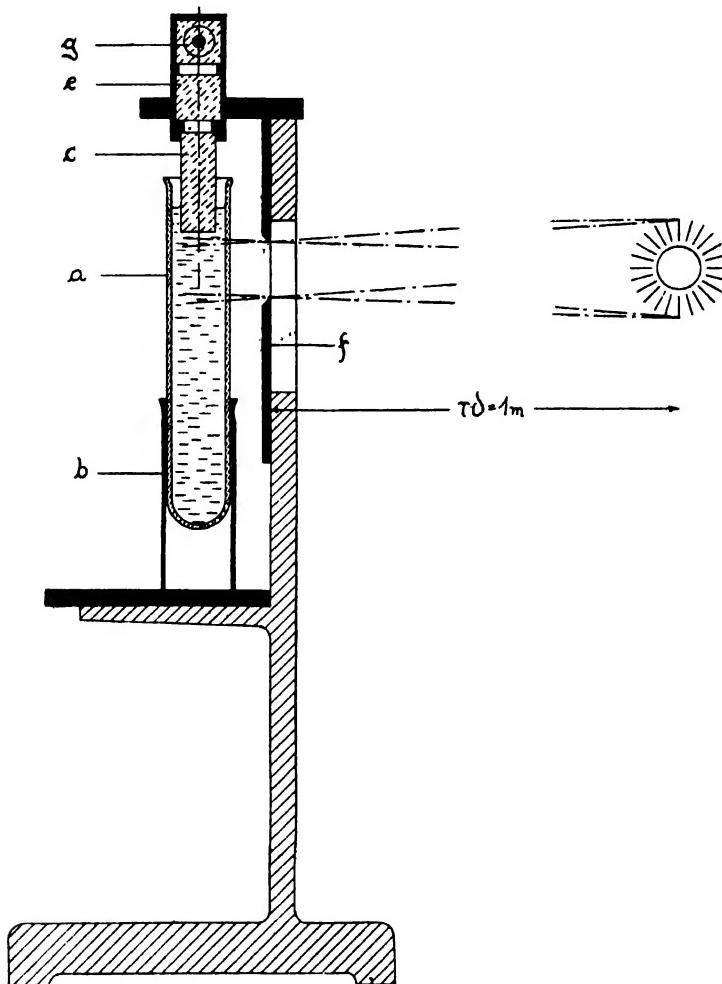
Fig. 9.—The Kleinmann Nephelometer—diagrammatic sketch of side view.
[from *J. Lab. Clin. Med.*, 12, 629 (1927)]

diameters than the cylinders ordinarily used, to fit the narrower test tubes and adapted to be fitted in their places by screw heads. A diaphragm Mi interposed in the path of the rays adapts the latter to the reduced diameter of the cylinders. (This diaphragm is now fitted in every instrument, so that the supplementary fitting for microanalysis may be used if desired without alterations in the instrument.)

Owing to the short radius of curvature of the test tubes R_1 and R_2 (**Figs. 11 and 12**), the light rays would on entering be so strongly deflected by refraction that the illuminated space, indicated in Figure 11 by a circle, would
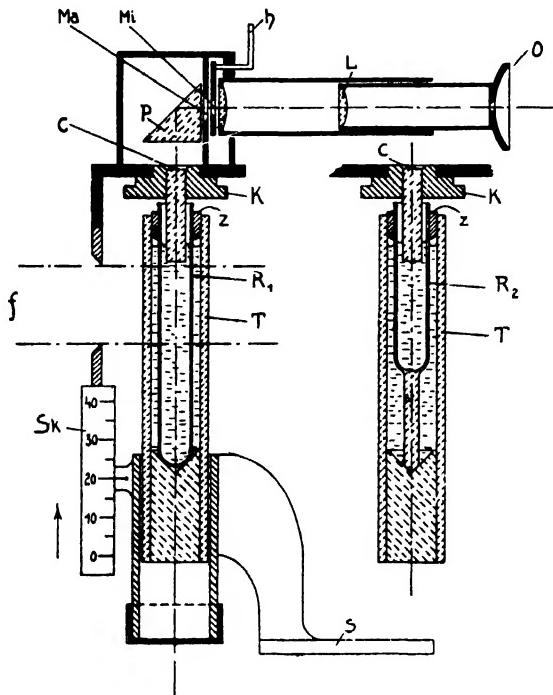
Fig. 10.—Kleinmann's Micronephelometer.
[from *J. Lab. Clin. Med.,* **12,** 629 (1927)]

become too small to yield sufficient light for observation. The test tubes are therefore enclosed in glass casings, which are filled with the solvent used in preparing the solution contained in the test tube. This arrangement (acting
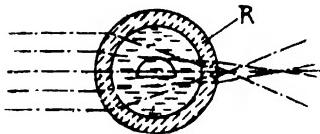
Fig. 11.   Fig. 12.
[from *J. Lab. Clin. Med.,* **12,** 629 (1927)]
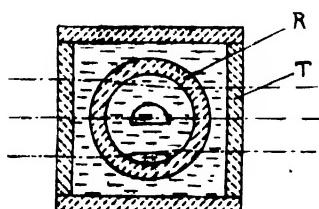
similarly to the immersion of the object glass of a microscope) diminishes the refraction, as is shown by Figure 12 and the path of the light rays is sufficiently extended to enclose the immersed cylinder.

Test tube and casing are supplied mounted, ready for fitting into the nephelometer. The disposition of the parts will be easily understood from Figure 10. By means of this, material which is procurable only in extremely small quantities, such as immunized sera and the like, can be subjected to investigation. The minimum quantity which can be estimated by nephelometric methods is thus reduced to one eighth of that hitherto required.

Protective Colloids. Since nephelometry depends upon the accurate comparison of the turbidities of two solutions (unknown and standard) it is obvious that nephelometric suspensions must be sufficiently stable to allow such a comparison. Some suspensions change only very slightly on standing, while others are so unstable that a "protector" or "stabilizer" is necessary in order to prevent, or at least decrease, their rate of coagulation and precipitation. For a further discussion of protective colloids see later on; also Vol. 1, p. 619.

General Conditions for the Production of Nephelometric Suspensions.[16] Our knowledge regarding suspensions is still very meager. Much work remains to be done regarding their production and their behavior, especially from a theoretical point of view. A few facts however have been gathered from the experience of those who have worked in this field.

Properties of Nephelometric Suspensions. Compounds which have an appreciable solubility in the media in which they are produced, are not suitable for nephelometric work. Nephelometric suspensions have thus far been produced in very dilute solutions, and a compound with an appreciable solubility, which may not be a large factor in ordinary gravimetric conditions, will in the dilutions required either not precipitate at all or only very partially. Of course, the medium in which the reaction takes place may in some cases be changed to make the compound more insoluble and nephelometrically suitable.

Therefore the first requirement of a compound for nephelometric determination must be great insolubility, or in other words the reaction producing the insoluble compound must be highly sensitive.

The second requirement is that of color, or rather the absence of color. Colored suspensions can be determined too, but as a rule they are measured by means of the absorbed light, i.e., colorimetrically, especially if the absorption of light is at all marked.

The exact chemical composition of the suspension in many cases is not known, but this although desirable and interesting, is not an essential matter, and does not enter into consideration in making a determination. Good nephelometric suspensions have been produced with almost all classes of chemical compounds; inorganic, organic, fat, oil, carbohydrate and protein suspensions have been produced. Therefore it is impossible to predict or limit the nephelometric behavior of any compound

Thus far the term nephelometric suspension has been used rather freely and therefore before proceeding with the conditions governing its production it may be well to define the term. By nephelometric suspensions are meant very insoluble suspensions produced in dilute solutions, (0.100 gram per liter or less), which show the same and constant amount of light on reflection, for a period sufficiently long to place them in a nephelometer and make the necessary number of settings with the instrument. This period varies with the substance, some suspensions show a change in the amount of light within

[16] From Philip A. Kober's chapter on Nephelometric Research in Yoe's "Photometric Chemical Analysis," Volume II (Nephelometry), John Wiley and Sons, Inc., New York   In press.

10 to 30 minutes, others only after one or two days. The change in the amount of light referred to, is usually a decrease in the amount of light and is caused by the growth in size of the particles in suspension, until agglutination sets in, so that the particles become macroscopically visible and settle out. Before this change takes place, they are homogenous to the naked eye and visible only with the microscope or ultra-microscope. In most cases this change, however, amounts to only 2 to 3 per cent of the light reflected before. To the observer it is apparent by a sudden jump in the readings, which before that time were constant.

This change in the amount of light, or the breaking down of the suspension, does not affect the accuracy of well developed nephelometric procedures, as the readings are easily made before this phenomenon occurs. Furthermore this change can be delayed for any reasonable time by the use of a protective colloid, which subject is discussed in the preceding chapter.

The third requirement for nephelometric suspensions is that the precipitation must be undertaken, as stated before, in a dilute solution. For most substances the concentration is 0.100 gram per liter, some even require a concentration considerably less than this.

At the first thought one would expect that nephelometric suspensions are obtainable only in one class of substances, namely colloids, but this is far from the truth. Harry Jones [17] in his book, published before the develop ment of nephelometric methods, states that "precipitation," meaning settling out of solution, "is not the natural condition in chemistry" and, he adds, "*is one of the most important phenomena in all chemistry,* and we are so familiar with precipitation from our analytical days, that we are accustomed to look upon it as the natural condition when a solid is formed as a result of a reaction between two solutions. We see from above [meaning some previous discussion] that such is not all the case. A moment's thought will show why this is true. When substances react, they react, we believe, molecule for molecule. The solid when first formed either has molecular dimensions or there are only a few molecules of the solid aggregated." He then concludes that "*the colloidal solution or at most the colloidal suspension is the natural condition of solid matter when first formed as the result of a reaction.*" *

In ordinary gravimetric precipitation the concentration is so great and the conditions made so, that the precipitate is in the form of a suspension only for a short period of time, so short that in most cases it escapes observation. But as stated before this period is greatly prolonged by dilution, the character of the solvent, and the presence of protective colloids or other stabilizers. The widespread application of nephelometry to so many different types of substances fully affirms Harry Jones' statement that the colloidal suspension is "the natural condition of a solid matter when first formed as the result of a reaction."

Calibration and Correction Curves. For a detailed discussion of the construction and use of calibration and correction curves see Yoe's "Photometric Chemical Analysis." Such curves are required when a Kober type instrument is employed; the Kleinmann instrument has been so designed that a correction factor is not necessary, at least as far as present applications have shown.

[17] "New Era of Chemistry," D. Van Nostrand Co., New York, 1913.
* von Weimarn long ago advocated this view. See his paper in Vol. I, this series. *J. A.*

Nephelometric Methods of Analysis

In the following pages a brief discussion of the principles of a number of nephelometric methods is given. New methods are being rapidly developed.

Acetone.[18] When acetone is added to a silver-mercury-cyanide solution an abundant white nebulous precipitate is produced. The reaction is so delicate that a solution of freshly distilled acetone containing 0.010 mg. per liter, i.e., 1 part in 100 millions, is sufficient to cause a distinct opalescence. Moreover, the intensity of the opalescence, as measured nephelometrically, has been found, within limits, to be proportional to the amount of acetone added. The method is adapted to the determination of very small amounts of acetone such as occur, for example, in a few cubic centimeters of normal blood.

Ammonia.[19] Nessler's reagent for ammonia, developed about the middle of the last century, was applied to water analyses in 1865. It has stood the test of time and has come to be used extensively; but with the development of colorimetry its disadvantages as well as its value have become apparent, and innumerable modifications of the reagent have resulted. Its instability and tendency to produce a cloud in dilute solutions are the chief difficulties.

Recently efforts have been made to apply the reagent in micro-Kjeldahl work, without previous distillation, with varying degrees of success; the precipitate due to salts makes the matching of colors, however, extremely difficult.

A probable explanation of why the colored solution produced by Nessler's reagent becomes cloudy, especially in the presence of salts, may be found in the following consideration:

(*a*) Only the iodide complex of mercury and ammonia is highly colored.

(*b*) The other complexes of mercury and ammonia, like the chloride (Na_2HgCl_4), are colorless insoluble compounds.

Therefore, in the presence of the other salts, the colored iodide complex is probably partially changed to and in equilibrium with the colorless complexes such as the chloride or sulfate.

On this basis Miss Graves has developed a sensitive nephelometric reagent for ammonia, consisting of mercuric chloride, sodium chloride, and lithium carbonate. The lithium carbonate was chosen on account of the low atomic weight of the cation and has, therefore, but slight tendency to cause agglomeration of suspensoids.

This reagent will detect 1.0 part of ammonia in 160 million parts of water and is useful in various tests and Kjeldahl nitrogen determinations. Moreover, this method saves considerable time as well as eliminates the expense, attention and errors connected with a battery of Kjeldahl stills.

Calcium. A. This nephelometric method is based essentially on the same principles used by Lyman [20] in his turbidimetric method for the determination of calcium in urine and feces,[21] the chief differences being that a solution of ammonium stearate is substituted for the castor oil soap and the clouds produced are matched in a nephelometer instead of a colorimeter.

The method is short, two hours being required for a set of four determinations as against three full days by the ashing and gravimetric procedure.

[18] Marriott, *J. Biol Chem.*, **16**, 289 (1913).
[19] Sara S. Graves, *J. Am. Chem. Soc.*, **37**, 1171 (1915); see also, P. A. Kober, *J. Ind Eng. Chem.*, **10**, 558 (1918).
[20] Lyman, *J Biol Chem.*, **29**, 169 (1917).
[21] Lyman, *J. Biol. Chem.*, **21**, 551 (1915).

Moreover, it requires only 5 cc. of blood instead of 300 cc. to 500 cc. and the results are accurate to within less than 1 per cent. The reagent will easily detect 1.0 part of calcium in 5 million of water and is useful in various tests and calcium determinations in blood, milk, water, etc.

B. This nephelometric method [22] was developed for the determination of small amounts of calcium in blood and other biological material. One cc. of blood should be enough for a determination but if Kleinmann's micro-nephelometer is used and the final dilution reduced, 0.25 cc should suffice. Calcium may be precipitated with oxalate to separate it from magnesium which is included in this determination. Possibly in blood analysis this may be dispensed with since the magnesium content of blood is small and probably constant in amount.

The reagent is sodium sulforicinate in dilute sodium hydroxide solution.

The sample containing 0.04 to 0.4 mg. calcium is ashed and the ash taken up in a few drops of N HCl, made alkaline with N NH$_4$OH, diluted to about 5 cc. and 0.4 cc. of the reagent added. After 3 minutes, dilute to 25 cc. and compare with a standard suspension. The error is 1 per cent.

Chloride. A. This method depends upon the opalescence obtained by adding silver nitrate solution to a solution containing a small amount of chloride ions. It was first used by T. W. Richards [23] in connection with some atomic weight determinations and was designed to estimate precipitates in amounts under 1 or 2 milligrams per liter of suspending solution.

B. This method [24] is identical in principle to the original one developed by Richards and Wells but differs in technique from the latter and appears superior in two respects, namely: (1) it develops a maximum and more constant opalescence (especially in the case of more concentrated solutions), and (2) it is simpler in that the same procedure is applicable over a wide range of concentration. The chloride in solutions ranging in concentration between 4 and 300 x 10^{-6}M can be determined with an average deviation of about 3 or 4 per cent.

The method was developed in connection with the study of "war gases" so very toxic that extremely minute amounts are significant. Even when large samples of the air-gas mixtures were collected (13 liters), it was necessary to quantitatively estimate chlorine as small as a few thousandths of a milligram.

The method of Richards and Wells had been applied only to aqueous solutions. In the analysis of toxic gases it was necessary to absorb them in alcoholic solutions and it was desirable to apply the method directly to this solution. After an extensive experimental study of the various factors involved, a procedure was finally obtained which satisfied all the requirements.

Dichloro-ethylsulfide (Mustard Gas). This method was developed during the World War by Yablick, Perrott and Furman.[25] It consists essentially in reducing a one per cent solution of selenious acid in 1:1 sulfuric acid, by means of dichloro-ethylsulfide vapor, to an orange-red suspension of selenium. The solution was heated to about 85° to facilitate reaction. As little as 0.005 mg. of the substance can thus be detected.

[22] Rona and Kleinmann, *Biochem. Z.*, 137, 157 (1923)
[23] *Z. anorg. Chem.*, 8, 268 (1895); Richards and Wells, *Am. Chem. J.*, 31, 235 (1904); *Proc. Am. Acad. Arts and Sci.*, 30, 385 (1894); *J. Am. Chem. Soc.*, 27, 484 (1905); Wells, *Am. Chem. J.*, 35, 99, 508 (1906); Richards, *ibid.*, 35, 510; *J. Chem. Soc.*, 99, 1204 (1911); *Orig. Com. 8th Intern. Congr. Appl. Chem.*, 1, 423 (1912); *ibid.*, 27, 24.
[24] Lamb, Carleton and Meldrum, *J Am. Chem Soc.*, 42, 251 (1920).
[25] *J. Am. Chem. Soc.*, 42, 266 (1920).

The amount of the suspension produced is nearly directly proportional to the amount of dichloro-ethylsulfide present. Amounts of the compound from 0.10 mg. to 0.01 mg. can be estimated nephelometrically with a maximum error of about 0.005 mg.

The method was developed to meet the need of a qualitative test for mustard gas, sufficiently delicate to indicate the presence of dangerous amounts of the substance in the air. An idea of the sensitivity required of the test is obtained when it is known that concentrations of mustard gas as low as 0.0005 mg. per liter (0.08 part per million) will cause discomfort on exposure for 25 minutes and concentrations between 0.001 mg. per liter and 0.005 mg. per
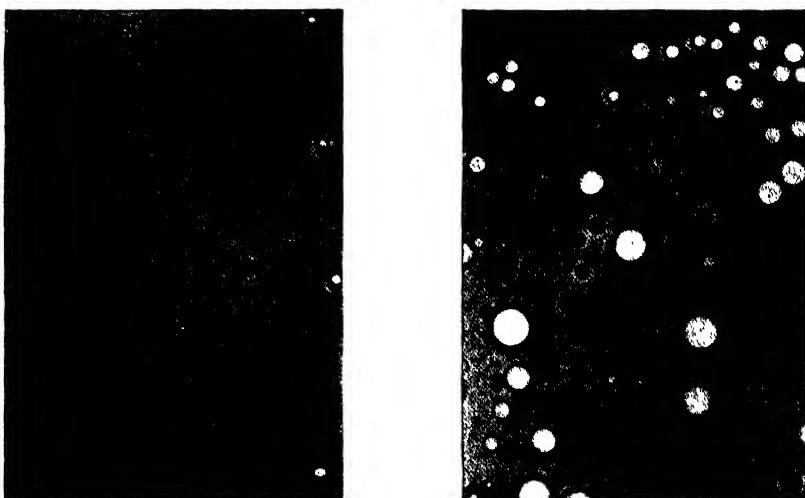
Fig. 13 - Photomicrographs of oil emulsions, > 525
Rona and Kleinmann *Biochem Z.* 174 25 (1926)

liter (0.2 p. p. m., and 0.8 p. p. m.) will cause skin burns if exposure is as long as 30 minutes.

The method has had a variety of uses, among which may be mentioned the detection of the presence of mustard gas in field and factory, securing of quantitative data as to the persistency of the gas over contaminated soil, and determining the permeability of protective fabrics to the vapor and liquid.

Fats and Oils. In quantitative work where a precipitate is easily thrown down, easily washed, dried and weighed, gravimetric analysis imposes no difficulty, although it may fail to estimate small quantities. Fats and oils are difficult to filter and to free from solvent and hence Bloor [26] devised a nephelometric method which avoids these difficulties. The fat or oil is extracted with an alcohol-ether solution and the extract poured into water. The fat or the oil separates out in fine globules forming a cloudy suspension. Bloor's method will easily estimate quantitatively 0.05 mg. of fat and will show the presence of 1.0 part of fat in a million of water. The method is

[26] *J. Biol. Chem.*, **17**, 377 (1914); *J. Am Chem. Soc*, **36**, 1300 (1914); see also, Kober, *J. Ind. Eng. Chem.*, **10**, 561 (1918).

useful in the nephelometric determination of fat in blood and in milk, and has been applied by Woodman, Gookin and Heath [27] to the nephelometric estimation of essential oils.

In Figure 13 are shown photomicrographs of oil emulsions magnified 525 times. Figure 14 shows oil emulsions photographed under the ultramicroscope.

Hydrolysis. A. Casein. Rona and Kleinmann [28] have used a nephelometric method to study the hydrolysis of casein by pepsin and trypsin. A solution containing more than 3 mg. of casein, per 5 cc. of 0.025 N sodium acetate,
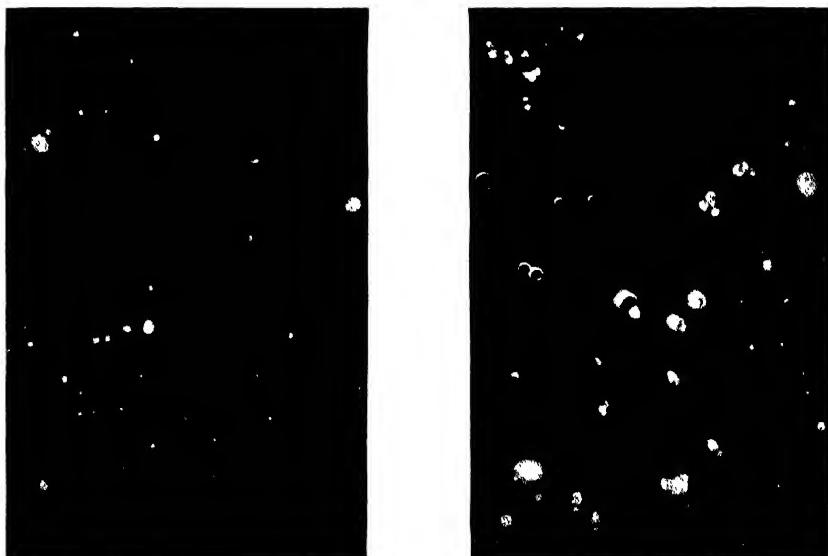
Fig. 14.—Photographs of oil emulsions under the ultramicroscope. Rona and Kleinmann, *Biochem. Z.*, 174, 25 (1926)

can be analyzed for casein by precipitation with a saturated quinidine-hydrochloric acid solution. From 20 to 30 cc. of the quinidine-hydrochloric acid solution must be used, the pH range must be 7.6 to 7.8, and the readings must be made within 10 to 45 minutes. Under those conditions, successive readings agree within 1 to 2 per cent.

B. Rate of Protein Hydrolysis. Rona and Kleinmann [29] have determined the rate of hydrolysis of proteins by pepsin at various hydrogen ion concentrations and in the presence of various electrolytes by means of the nephelometer. The optimum pH for hydrolysis is 2.1-2.2. The ions Na, K, Ca, Cl, NO_3 and SO_4, in more than very small concentrations, hinder the hydrolysis. On the alkaline side of the optimum pH, small concentrations of salts may favor the hydrolysis.

Nucleic Acids. This method was developed by Kober and Graves [30] for

[27] *J. Ind. Eng. Chem.*, 8, 128 (1916).
[28] *Biochem. Z.*, 155, 34 (1925).
[29] *Biochem. Z.*, 150, 444 (1924).
[30] *J. Am. Chem. Soc.*, 36, 1304 (1914).

the purpose of estimating undigested nucleic acids. It consists in adding a 0.2 per cent solution of acid egg albumin to dilute solutions of nucleic acids and estimating nephelometrically the resulting suspensions. This reagent will easily detect one part of nucleic acid in a million parts of solution and is not appreciably affected in dilute solutions by most substances met with in physiological work.

β-Oxybutyric Acid.[31] The β-oxybutyric acid is oxidized to acetone and the latter determined nephelometrically by means of Marriott's silver-mercury-cyanide solution.

The method is useful in estimating β-oxybutyric acid in blood and tissues. Since oxidation of β-oxybutyric acid by chromic acid gives only 90 per cent of the theoretical yield of acetone, 10 per cent should be added to the results thus obtained.

Phosphate.[32] This determination is based upon the strychnine-molybdic acid colorimetric reagent of Pouget and Chouchak,[33] except that the reagent is made up in hydrochloric acid instead of nitric acid. The nephelometric reagent remains practically colorless for an indefinite length of time, is stable and gives quantitative and constant results. It will detect 1.0 part of phosphorus in 333 million parts of water and 0.005 mg. of phosphorus in 10 cc. of solution, i.e., 1 part of phosphorus in 2 million parts of water, can easily be estimated quantitatively with the nephelometer.

The reagent is useful in various tests and in phosphorus determination in urine, iron, steel, etc.

Kleinmann[34] has also developed a nephelometric method for phosphate. His method is applicable to amounts from 0.1 to 0.0005 mg. P_2O_5, with an error of only 0.5 per cent.

Proteins. The quantitative estimation of proteins and other colloidal substances is usually a long, tedious, and often inaccurate process. Such substances form suspensions that are extremely difficult to filter and require much washing to remove the small amount of adsorbed mother-liquor. Moreover, some precipitates are quite soluble in the wash water. By means of the nephelometer such substances can be accurately and rapidly determined quantitatively, provided a suitable precipitant can be found. By "suitable precipitant" is meant a substance that will produce a quantitative precipitation in solutions 0.01 per cent or weaker and in the form of a suspension which does not agglutinate appreciably in less than 10 to 20 minutes, i.e., time enough to permit taking two to five readings with the nephelometer. Kober[35] has obtained such precipitants for the proteins. He employs a 9 per cent sodium chloride solution for edestin and a 3 per cent solution of sulfosalicylic acid for albumins, globulins and native proteins, i.e., coagulable proteins.

From two to three days are required for the determination of casein, globulin and albumin in milk when it is done by the usual technic, whereas with the nephelometric method it can be done in twenty to thirty minutes.

Kober's nephelometric method of estimating proteins will show the presence of 1.0 part of protein in a million parts of water.

[31] Marriott, *J. Biol. Chem.,* **16**, 295 (1913).
[32] Kober, and Egerer, *J Am Chem. Soc.,* **37**, 2373 (1915).
[33] *Bull. soc. chim.,* **5**, 104 (1909); **9**, 649 (1911).
[34] *Biochem. Z.,* **99**, 150 (1919).
[35] *J. Biol. Chem.,* **13**, 485 (1912-13); *J. Am. Chem Soc.,* **35**, 290, 1585 (1913); see also Rona and Kleinmann, *Biochem. Z.,* **140**, 461 (1923).

Purine Bases, Including Uric Acid. Salkowski's reagent for purine bases, which consists of equal volumes of magnesia mixture and ammoniacal silver nitrate (26 g. per liter and sufficient NH_4OH to prevent precipitation of Ag_2O), has been modified by Graves and Kober [36] to meet nephelometric conditions. The ammoniacal silver nitrate precipitates the purine bases as a white silver complex, the presence of chlorides in the reagent preventing the reduction of the silver salt to black metallic silver by uric acid.

The modified reagent contains a lower concentration of silver nitrate, and ammonium chloride alone instead of magnesia mixture. It will precipitate xanthine, hypoxanthine, guanine, adenine and uric acid quantitatively in solutions as dilute as 0.0002 per cent. By use of egg albumin as a protective colloid the precipitates are kept in suspension and may be estimated nephelometrically.

In a mixture of purine bases, including uric acid, the latter may be oxidized quantitatively in 3 to 5 minutes by means of a suspension of manganese dioxide and without any appreciable effect on the four purine bases— xanthine, hypoxanthine, adenine, and guanine. Hence, to estimate uric acid, and the other purine bases separately, it is only necessary to determine (1) the total purine content and (2) the purine bases (excluding uric acid). The difference between (1) and (2) will give the uric acid content.

Sulfate. The method developed by Denis [37] for the determination of inorganic sulfate in blood, consists in the removal of protein by means of a solution of mercuric chloride and hydrochloric acid, the formation of a colloidal suspension of barium sulfate in the filtrate, and the nephelometric measurement of the suspension.

Colorimetry

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If we classify methods in analytical chemistry according to the physico-chemical principles involved, it will be found that practically all quantitative procedures may be grouped under the following important principles [1]: (1) Neutralization, (2) Solubility-product, (3) Oxidation-Reduction, (4) Photometric Chemical Analysis (Colorimetry and Nephelometry), and (5) Evolution and measurement of gases. In this paper and the succeeding one we are concerned with the fourth principle. *Photometric chemical analysis* may be defined as *analysis which depends upon a change in the amount or character of light due to a chemical reaction.* The change in the amount of light as understood in this and the following paper, is that due to either absorption or reflection. Analysis made on the basis of absorption is usually called *colorimetry* and in a few cases turbidimetry, while analysis based on reflected light is called Nephelometry. We shall treat Colorimetry in this paper and Nephelometry in the succeeding one.

The use of color as a means of determining the amount of a given substance present has long been employed, for example, the determination of ammonium, nitrite, and nitrate nitrogen in water, or of carbon in steel. Also, color may be referred to an absolute index of color value, for example, by use of the Lovibond tintometer; or it may be determined by absolute analysis in terms of wave length of dominant hue or its complement and the percentage of white, for example, monochromatic analysis by means of the Nutting colorimeter.

Colorimetric methods have rapidly increased in number during the past twenty-five years, so that the list now includes many metals (aluminum, chromium cobalt, copper, gold, iron, lead, magnesium, manganese, mercury, molybdenum, nickel, potassium, tin, titanium, tungsten, vanadium, zinc, etc.), many non-metals (arsenic as arsenate; boron as borate; carbon: hydrogen-ion; nitrogen as nitrite, nitrate, and ammonium; oxygen both free and as hydrogen peroxide; sulfur as sulfide and as sulfate; phosphorus as phosphate; etc.), and a large number of organic substances which include nearly all classes of organic compounds (alcohols, aldehydes, organic acids, esters, phenols, carbohydrates, alkaloids, hemoglobin, etc.).

In general, colorimetric analysis consists in adding a reagent to a solution of the test substance in such a way as to produce a color. The basis of colorimetry may be stated as follows: When equal heights or thicknesses of two solutions give the same intensity of color, the concentration of the solutions are said to be equal. When equal color intensity is obtained from different heights of two solutions, the assumption is often made in accordance with

[1] *Cf.* H. A. Fales, "Inorganic Quantitative Analysis," p. 5, The Century Co., New York, 1925.

Beer's law—that the concentrations are inversely proportional to the heights. While many colors follow Beer's law sufficiently closely, recent colorimetric work makes no assumption at all, but the amount of color for each concentration is obtained by standardization with each color under the conditions found in any particular determination.*

Colorimetric Apparatus

Colorimetric apparatus may be divided into two types according to the method of comparison:

1. Apparatus used in the (*a*) standard series, (*b*) dilution, and (*c*) duplication methods.
2. Apparatus used in the balancing method.

Apparatus of the first type is generally very simple, consisting of bottles, Nessler tubes, Eggertz tubes, Julian tubes, the color camera, etc. The balancing method requires more elaborately constructed apparatus, but in use is simplest. These instruments range from the simple Hehner cylinders to the elaborate plunger type and wedge type colorimeters, having special optical arrangements which enable high precision in matching colors.

The plunger type colorimeter with the two halves of the field of view illuminated by the light passing through the unknown and standard solutions respectively, was first announced by Jules Duboscq, of Paris, in 1854. Improved modifications of this instrument have been made in recent years by various manufacturers, particularly in the United States.

Special forms of colorimeters, some of which are less elaborate and less expensive than the Duboscq type, have been developed. Among these may be mentioned the Schreiner colorimeter for soil work; the Kennicott-Campbell-Hurley colorimeter for water analysis, rock analysis, steel analysis, analysis of alloys, etc.; the Saybolt chromometer for oil testing; the Stammer colorimeter used in sugar analysis for grading syrups, and estimating the decolorizing power of bone black and other clarifying agents, and for many other purposes for which the degree of color, and not determination of color-producing substance, is desired; and the Lovibond tintometer for standardizing merchantable petroleums and for other purposes.

The Duboscq, Bausch & Lomb-Duboscq, Leitz-Duboscq, Spencer-Duboscq, Kober, Bock-Benedict, Schmidt & Haensch, and Kleinmann colorimeters, are designed particularly for biochemical and clinical work, such as the determination of creatinine, total nitrogen, and urea in urine, etc., and the determination of non-protein nitrogen, urea and ammonia in blood. These are instruments of high precision and are useful for work requiring the highest degree of accuracy, especially when only a small amount of sample is available.

Colorimeters of the wedge type are illustrated by White's colorimeter, which is suitable for the analysis of ores and alloys containing fairly large amounts of the test substances, and by Myers' bi-colorimeter (three-wedge type) which has been constructed primarily for the determination of hydrogen-ion concentration.

Even brief descriptions of a number of the many colorimeters now used would be out of place here. We shall, therefore, limit ourselves to a short

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* For a detailed discussion of colorimeter calibration and correction curves see Chapter IV in Yoe's "Photometric Chemical Analysis," John Wiley and Sons, Inc., New York, 1928.

description of the latest model of the Duboscq colorimeter as manufactured by the Bausch & Lomb Optical Company. This colorimeter is an instrument of precision, designed to meet the most exacting requirements of the analyst. Into its construction are built such refinements as optically inactive tube bottoms, plungers of optical glass matched for color, adjustments of microscopic precision and a dust-proof housing for the prism system.

Essentially the Duboscq colorimeter is a high-grade photometric instrument. Light from some even source of illumination is passed through the two sides of the instrument. There are interposed in these two light paths the substances which are to be tested. In passing through the liquids some of the light is absorbed, the amount of absorption depending on the depth of the solution. The two beams of light are now brought to a common axis by
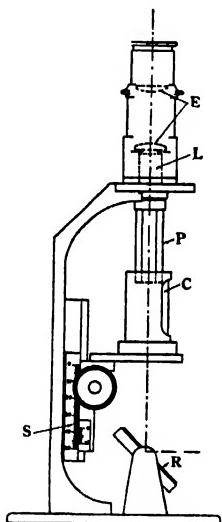
FIG. 1.
(Side view)
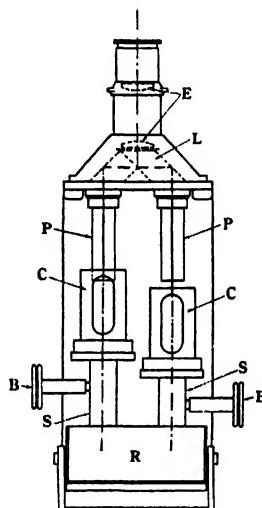
FIG. 2.
(Front view)

Bausch & Lomb Colorimeter.

means of the rhombohedral prisms. Light from one cup illuminates one-half of a circular field and light from the other cup illuminates the other half. The observing microscope, by which the observer sees both fields with one eye, is focused on the line of separation of the two fields. It is now possible to alter the depths of the two columns of liquid until the two halves of the field are identical in intensity. When this condition holds, the concentrations of the two solutions are inversely proportional to the depths (provided Beer's law holds, otherwise a correction curve is used), which are read on the scales at the side of the instrument. The instrument is illustrated diagrammatically in Figures 1 and 2.

In the following pages we shall limit ourselves to a discussion of the rôle of colloids and colorimetric stabilizers in colorimetry.

Colloids and Colorimetric Stabilizers

In most colorimetric methods of analysis we deal with true solutions whose colors are due either to the presence of colored molecules or to colored ions. That is to say, the component substances of the solutions are molecularly (or ionically) dispersed in each other. Some methods, however, are based upon the formation of colored colloid suspensions. These suspensions, or so-called colloidal solutions, are in some cases fairly stable and may be compared in a colorimeter in the usual way without there being a serious error introduced, due to aggregation of the particles. On the other hand, some of the suspensions are so unstable that the color matching must be made very rapidly, and great care must be taken to prepare the "unknown" and "known" simultaneously in order to secure approximately the same ageing effects.

Protective Colloids and Other "Stabilizers." In the case of finely divided suspensions it is possible to increase their stability, i.e., prevent or at least decrease their rate of coagulation and precipitation, by adding a *protective colloid* or stabilizer such as gelatin, gum arabic, starch, egg albumin, etc.

It was observed as early as Faraday [2] (1857) that the addition of a small amount of "jelly" (probably gelatin) prevented the coagulation and precipitation of metal sols. Such organic substances as gum arabic, gelatin and the proteins are themselves largely colloidal in solution and since they stabilize and protect from coagulation unstable sols, they have been termed "protective colloids." It is held that they form a film around the suspended particles in a sol and thus protect them from coalescence. For example, arsenious sulfide sol containing a little gelatin is not coagulated by the addition of electrolytes in amounts much greater than would cause precipitation if no protective colloid were present. This protective action is known as the *envelope theory of protection* and was enunciated by Bechhold.[3] The mechanism of such action has been quite definitely demonstrated by Jacques Loeb [4] by a comparison of the stability of protein solution with that of dispersions of protein-coated collodion particles.

The protective power of these hydrophilic or "protective" colloids varies greatly and is measured in terms of the "gold number," a term due to Zsigmondy.[5] The "gold number" is the maximum number of milligrams of protective colloid that may be added to 10 cc. of a standard gold sol without preventing a change of color from deep red to violet shades by 1 cc. of a 10 per cent sodium chloride solution. It must be remembered, however, that the "gold numbers" are useful solely as very rough indices of relative protective powers, because the gold number of a given protective colloid depends so largely upon many conditions. Lack of space prohibits discussing here further aspects of this subject.

In addition to stabilizers of the above type, there are those of the "solution link" type, e.g., H_2S, $FeCl_3$, etc., described by Thomas and Frieden.[6] Examples of colorimetric methods involving such stabilizing action are: the sulfide methods for lead, copper, iron, and antimony in which H_2S is the stabilizer. For a theory of the mechanism of such stabilizing action, see the paragraphs on *"The Solution Theory of Colloid Stability," vide infra.*

[2] Michael Faraday, *Phil. Trans.*, 147, 184 (1857).
[3] *Z. physik. Chem.*, 48, 385 (1904)   Many other colloids function as protectors, e.g. in purple of Cassius, colloidal stannic acid protects gold. *J. A.*
[4] *J. Gen. Physiol.*, 5, 479 (1923).
[5] *Z. anal. Chem.*, 40, 697 (1901).   See also paper by J. Alexander in Vol. I of this series   *J. A.*
[6] A. W. Thomas and A. Frieden, *J. Am. Chem. Soc.*, 45, 2522 (1923); Thomas, *J. Chem. Education*, 2, 323 (1925).

Some examples of the use of hydrophilic or protective colloids in colorimetric analysis are: (1) gum arabic in the determination of antimony as the sulfide, (2) gum arabic in the determination of bismuth as the iodide, (3) gum arabic in the determination of selenium, (4) gelatin in the determination of lead as the sulfide, (5) gelatin in the determination of acetylene by ammoniacal cuprous chloride, and (6) gum arabic in the determination of phenols in blood.

Yoe and Hill [7] have recently shown that the addition of a small amount of starch solution to a solution containing aluminum gives a stable suspension of the deep red aluminum lake produced by the addition of the dye aurin tricarboxylic acid. The use of starch solution permits determining colorimetrically aluminum in much higher concentrations than would be possible without the presence of a protective colloid to stabilize the colored suspension. Moreover, the presence of this protective colloid does not impair the sensitivity of the determination. This latter statement must not be taken to imply that such would *always* be the case with other constituents or with other protective colloids. In each case precaution must be taken to determine what effect, if any, the presence of a given protective colloid has.

The effect of protectors on the color changes of benzopurpurin when its solutions are acidified, has been studied by Jerome Alexander.[8] He observed that "in the case of a pure dilute solution of benzopurpurin, the addition of dilute mineral acids quickly changed the color from bright red to dark blue, reminding one of the change of pure colloidal gold. Stronger acid coagulated the dye, which settled out of solution but which could be redissolved with restoration of the original color, by neutralizing the acid with alkali. The same solution of benzopurpurin, to which gelatin or gum arabic had been added, gave with dilute mineral acids a claret-red solution. More concentrated acid changed the shade to chocolate-brown, without, however, causing any precipitate." See also the following paragraphs on the effect of emulsoids on colored solutions.

Effect of Emulsoids on Colored Solutions. "The color of the solution of a colored substance of small molecular weight is little or not at all affected by the presence of an emulsoid which does not react chemically with the colored substance and which does not change the hydrogen ion concentration of the solution. When the colored substance has a large molecular weight, the presence of an emulsoid may influence the color of the solution in a number of ways. Should the colored solution be a suspensoid, the emulsoid will protect it from coagulation, as for example, gelatin protects colloidal gold solution. Should an insoluble colored substance be liberated from its soluble form the emulsoid will prevent its precipitation, as is the case when an acid solution of Nile blue is made alkaline in the presence of egg albumin. Finally, if the colored substance happens to fade on standing, the emulsoid may retard the rate of fading. This is true of methyl violet and gum arabic in alkaline solution. In all such cases the emulsoid exerts its influence by retarding or preventing some change which would take place in its absence, and consequently the color of the solution containing the emulsoid will differ from that of the same solution containing no emulsoid." [9]

[7] Yoe and Hill, *J. Am. Chem. Soc.*, **49**, 2395 (1927).
[8] *J. Soc. Chem. Ind.*, **30**, 517 (1911); also Seventh International Congress of Applied Chemistry and Vol. I of this series.
[9] H. Wu and D. Y. Wu in J. Alexander's "Colloid Chemistry," Vol. I, p. 380, The Chemical Catalog Co., New York, 1926

In addition to the above influence of emulsoids on the color of solutions, there is one which depends on an equilibrium between the emulsoid and the colored substance. In connection with the colorimetric determination of hemoglobin as alkaline hematin, H. Wu and D. Y. Wu attempted to use as the standard of comparison pure alkaline hematin solution prepared by dissolving crystalline hematin in alkali. They found, however, that the color of the pure hematin solution was much weaker than that of the solution prepared from the corresponding amount of hemoglobin. The only difference beween the two solutions was that the one prepared from hemoglobin contained a protein, globin, while the other did not. Evidently the globin increased the color of the alkaline hematin. Serum, egg white, gelatin and other proteins added to pure hematin solution gave similar results, though differing quantitatively. Other emulsoids such as gum arabic, starch, agar agar, etc., were tried, and all of them were found to influence the color intensity of the alkaline hematin. Another colored substance, Congo red, gave similar results.

"The influence of emulsoids on the color of alkaline hematin and Congo red solutions cannot be ascribed to a change in the alkalinity, for such a change of alkalinity as might be caused by the addition of the emulsoid was shown to have no appreciable effect in itself on the color of the solution. Moreover, the color of alkaline hematin and Congo red produced on the addition of an emulsoid, cannot be obtained in its absence by mere change of reaction. Hematin and Congo red are acids and they should not combine with protein, which in alkaline solution also behaves as an acid. Chemical combination of hematin and congo red with the polysaccharides is even more unlikely. That emulsoids of entirely different nature, such as egg albumin and gum arabic are, should exert a similar influence on the same colored substances, shows that the influence is physical and not chemical."[10]

Wu and Wu point out that there are two ways in which a colored solution may be influenced physically by an emulsoid, namely, (1) by adsorption of the colored substance by the emulsoid, and (2) by adsorption of the emulsoid by the colored substance. In the former case a layer of the colored substance is formed around the particles of the emulsoid, while in the latter case the reverse is true. The color of the "adsorption complex" in either case may differ in intensity and in shade from that of the particle of the free colored substance. The so-called "protein error" has long been recognized in the colorimetric determination of hydrogen ion. From the work of Wu and Wu it is evident that the presence of emulsoids is a factor to be considered in colorimetry in general.

The "Solution Theory of Colloid Stability"

"The origin of the solution forces and the reason for the electrical migration of colloidal dispersions of apparently insoluble substances such as ferric oxide, antimony sulfide, gold, etc., have gradually become apparent through years of research. It is now definitely known that these insoluble substances do not exist as such in colloidal solution. The particles in colloidal ferric oxide solution consist of a complex of Fe_2O_3 and a soluble iron salt such as $FeCl_3$; antimony sulfide hydrosol consists of a combination between Sb_2S_3 and H_2S.

[10] H Wu and D. Y. Wu, *loc. cit.*, p. 380.

Formulas may be roughly written for them (ignoring the hydration water) as follows: xFe$_2$O$_3$.yFeCl$_3$; xSb$_2$S$_3$.yH$_2$S where x and y are variable and x is always greater than y. When an electric current is passed through these solutions, a brown precipitate of Fe$_2$O$_3$ settles out at the cathode and chlorine is evolved at the anode in the iron oxide hydrosol, while in the antimony sulfide sol, a red deposit of Sb$_2$S$_3$ is deposited at the anode and hydrogen gas is liberated at the cathode. This shows that the migrating ions are:

xFe$_2$O$_3$.yFe^{+++}	$3y$Cl$^-$
$2y$H$^+$	xSb$_2$S$_3$.yS$^-$
To cathode	To anode
←	→
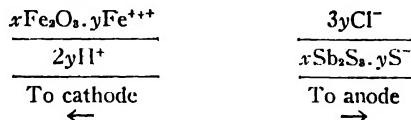
The ionization is not complete; it is in fact slight and the nature of the migrating ions is not quite so simple as indicated.[11] But it is not possible to make fine distinctions in a short discussion.

"The gradual realization of the fact that the "impurities," e.g., the FeCl$_3$ or the H$_2$S, were essential parts of certain hydrosol particles, gave rise to the so-called *complex theory,* a very simple statement of the complex nature of certain colloids.

"While the complex theory was accepted for many colloidal dispersions, hydrosols of noble metals such as gold and platinum were thought to be exceptions since it was believed that they could be prepared by electrically arcing these metals under pure water. This was disproved by Beans and Eastlack,[12] who demonstrated that colloidal platinum could be formed in pure water due to the fact that platinum oxidizes in the arc thus generating an electrolyte which became part of the dispersed phase. Gold was shown to require the presence of minute amounts of certain salts, in fact those which form stable chemical compounds of gold.*

"It is therefore not difficult to see the origin of the solubility forces, since insoluble substances in colloidal solution are actually a part of a complex aggregate containing a soluble component. Evidence for *solution* forces as the reason, or at least one of the reasons for colloid solution stability has been given by Thomas and Frieden.[13] A simple experiment may be cited. Addition of alcohol followed by ether to a hydrosol of xFe$_2$O$_3$.yFeCl$_3$ did not affect it. Alcohol promptly precipitated a hydrosol of xFe$_2$O$_3$.yFe$_2$(SO$_4$)$_3$. Ferric sulfate is insoluble in alcohol."[14] **

Interference Eliminators. Another class of agents employed in colorimetric analysis may be properly termed "interference eliminators." For example, in the determination of ammonia by Nesslerization, calcium and magnesium

[11] See J. Duclaux, *J. chim. phys.,* **7,** 405 (1909).
[12] Beans and Eastlack, *J. Am. Chem. Soc.,* **37,** 2667 (1915).
* See papers by A. de G. Rocasolano in Vol. I of this series, and paper by Wo. Pauli in this volume.
** These formulas give too simple a picture and are liable to mislead. For instance, the iron oxide some time without them. Recent work at the University of Wisconsin shows how to prepare stable sols of ferric hydroxide, *free* of chlorine. See paper in *J. Am. Chem. Soc.* (1928) by Dr. G. Harvey Sorum, J. A.
[13] A. W. Thomas and A. Frieden, *J. Am. Chem. Soc.,* **45,** 2522 (1923).
[14] A. W. Thomas, *J. Chem. Education,* **2,** 323 (1925).
* These formulas give too simple a picture and are liable to mislead. For instance, the iron oxide sol written as xFe$_2$O$_3y$FeCl$_3$ may give the idea that all the FeCl$_3$ is free to ionize in solution or that just the Fe^{+++} is adsorbed. For academic purposes a better simplified picture is (xFe$_2$O$_3$, wH$_2$O, yFeCl$_3z$Fe^{+++})$3y$Cl$_3$$^-$ where y is smaller than x, and z is very small in comparison with y. Private communication from Dr. Arthur W. Thomas.

are precipitated by Nessler's reagent and, hence, would interfere with the analysis. They may be removed by precipitating in the usual way and filtering, but it is more convenient to add Rochelle salt which holds them in solution. Another example of the use of an interference eliminator is in the determination of phosphorus as phosphomolybdate. Here, two or three drops of ammonium oxalate are added before adding the phosphate reagent in order to prevent the calcium precipitating as calcium phosphate.

Nesslerization. The yellow to brown color produced by the reaction of ammonia with Nessler's reagent is fairly stable and (when the ammonia concentration is not too high) gives a solution which appears water-clear and homogenous to the naked eye. However, Robertson and Hisey [15] have recently made an extensive experimental study of the nature of the Nessler color and conclude that it is due to colloidally dispersed particles in suspension and not in true solution.
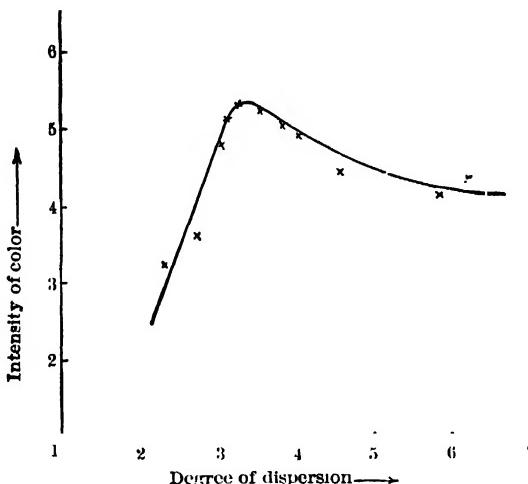
Fig. 3.—Relation of color intensity of colloidal gold to its degree of dispersion (according to The Svedberg).

[by permission, from Ostwald Fischer "Theoretical and Applied Colloid Chemistry," John Wiley & Sons, Inc., New York, **1922**, 2nd ed., p. 63]

Peptization. Finely divided particles [16] may be obtained either by condensation from vapor or solution (precipitation) or by disintegration of larger masses. The disintegration is frequently called *peptization,* especially if it is done chemically as opposed to mechanical or electrical disintegration. The word *peptization* is due to Thomas Graham.

In a study of the reduction of certain vat dyes by alkaline sodium hyposulfite, Yoe and Edgar [17] employed a colorimetric method of measuring the amount of dye reduced. Their results indicated that the reaction between the dye and the alkaline hyposulfite is very rapid, that an insoluble crystalline reduced dye (dark blue) is first formed, and that the latter is then *peptized by hydroxyl ions to form a dark blue sol.* The dark blue sol was matched in

[15] Private communication from Dr. J. H. Robertson.
[16] *Cf.* W. D. Bancroft, "Applied Colloid Chemistry," McGraw-Hill Book Co., New York, **1921**, p. 162.
[17] Yoe and Edgar, *J. Phys. Chem.,* 27, 65 (1923); Yoe, *ibid.,* 28, 1211 (1924).

a colorimeter against a standard sol similarly prepared, or against a standardized blue glass. The *rate* of peptization, and the *amount* of *dye stuff* peptized by a given solution, depend upon the state of subdivision of the dye. The rate of reduction is much faster than the rate of peptization.

Color and Degree of Dispersion. It has been shown by experiment that the color intensity of substances varies with the degree of dispersion and that it *attains a maximum in the realm of colloid dispersion.* It is well known that colloidal solutions of certain salts, e.g., the sulfides of lead, copper, arsenic, etc., show such a marked color, even in very low concentrations, that this property may be used for their recognition. The intensity of color of these colloidal solutions may at times exceed that of aniline dyes. For example, if the color intensity of fuchsin be represented by an arbitrary value of 5, that of colloidal hydrous ferric oxide is about the same, while that of arsenious trisulfide is 100, and that of colloidal gold about 2000 (The Svedberg).[18] This variation in color intensity is strikingly illustrated in the case of colloid gold. See Figure 3. The great variation in color phenomena exhibited by colloid metals was observed by Michael Faraday three quarters of a century ago, and he pointed out that the degree of dispersion is largely responsible for color formation.

This color variation with the size of the particles is also exhibited by certain dyes. For example, the color changes in Congo-rubin sols. "The particles of this dye sol have diameters between those of colloids and molecular dispersoids. It may be suddenly transformed to a blue-violet or blue solution not only upon addition of acid but also by the addition of any neutral salt or even alkaline substances. The dye behaves like a red gold sol in many respects and it may be used as a gold sol substitute. The color transition of Congo-rubin is reversible by dilution, by raising the temperature, by addition of alcohol, etc."[19] Another illustration of color change is the dye benzopurpurin, already mentioned. Ultramicroscopic observations by Alexander[20] showed that aggregation of the colloidally dispersed dye is accompanied by a change in color. This is no doubt true in the case of many other dyes.

Not only does the intensity of color vary with the degree of dispersion, but also the beauty and *variety.* "The *order* in which the colors change from one to the other as the degree of dispersion changes seems also to be definite. As a rule, the most highly dispersed colloid metals are yellow or orange; in other words, they absorb violet and blue light. As the degree of dispersion decreases, the color passes from yellow through orange to red, violet, blue and finally green. The absorption maximum gradually moves towards the side of the greater wave lengths as the degree of dispersion decreases."[21]

It is natural that color intensity of metals (especially the so-called noble metals) should have been called upon for analytical purposes, and is not surprising that one of the oldest and best known methods for demonstrating the presence of traces of gold consists in reducing the gold to the colloid condition. The *purple of Cassius* test for gold is a typical illustration of the production of gold in the colloid state of division and its subsequent

[18] *Cf.* Ostwald-Fischer, "Theoretical and Applied Colloid Chemistry," John Wiley and Sons, Inc., New York, **1922**, 2 ed., p. 63.
[19] Wo. Ostwald, et al., "Practical Colloid Chemistry," E. P. Dutton and Co., New York, **1927**, (Translation of 4 ed.), p. 70.
[20] *J. Soc. Chem. Ind.*, **30**, 517 (1911).
[21] Ostwald-Fischer, *loc. cit.*, p. 65. For further details regarding this relation between color and degree of dispersion, see Wo. Ostwald, *Kolloidchem. Beih.*, **2**, 409 (1911) and "Licht und Farbe in Kolloiden," Th. Steinkopff, Dresden and Leipzig, **1924**.

precipitation in the form of an "adsorption compound." The first step in this test is accomplished by reducing the gold salt with stannous chloride. In this way colloid gold and colloid stannic acid are formed, and these in turn unite to form the well-known, reddish-violet colored solution or precipitate.

Concentration by Adsorption on a Crystalline Solid. Another application of colloid chemistry to colorimetry is made in the case of the determination of zinc by the potassium ferrocyanide method.[22] This is a turbidimetric method described by Breyer in which zinc is precipitated as a colloidal suspension by potassium ferrocyanide solution. Meldrum has applied this method to the determination of zinc in water[23] and Birckner to the determination of zinc in various food products. Bodansky[24] modified the Breyer-Birckner method and used it to determine the zinc content of marine organisms. Solid calcium citrate is employed in the recovery of the colloidal zinc sulfide. A better recovery is obtained when the calcium citrate is formed in the solution, than when it is added pre-formed. It is thought that the calcium citrate adsorbs the colloidal zinc sulfide particles and, hence, effects a better recovery. This is in line with Bancroft's observation:[25] "There is some evidence to show that when a colloidal solution is precipitated, the finer particles attach themselves to the coarser ones."[26] The zinc sulfide is finally dissolved and reprecipitated as a finely divided suspension by the addition of potassium ferrocyanide.

Concentration by Co-Precipitation with a "Collector."[27] In the colorimetric method (thiocyanate method) of Stokes and Cain for the determination of iron, by far the greater number of cases require concentrating the iron by precipitation. An almost indefinitely small quantity of iron may thus be determined in an indefinitely large amount of material, the only limit being the solubility of the iron precipitate in the solution. It is obviously impossible to collect on a filter, traces (say a thousandth of a milligram) of ferric hydroxide or sulfide distributed in a finely divided state through a considerable volume of an otherwise clear liquid. Stokes and Cain therefore employ the method which has been occasionally used successfully in other cases,* of mechanically carrying down the minute amount of finely divided precipitate by a relatively large amount of another precipitate, which when practicable, is generated simultaneously with the iron precipitate. We may designate this secondary precipitate as the "collector." Various substances suggest themselves as collectors; their number is limited by the following considerations. A collector must be sufficiently insoluble, so that but a small amount of a possible impure foreign substance need be introduced; it must be of such physical consistency as to enable it to carry down all suspended precipitates, and must therefore be amorphous and flocculent, not granular or crystalline; it should not be gelatinous or otherwise difficult to wash out in the filter, neither should it be of such consistency as to run through the filter on washing; it must be easily soluble in 7 per cent thiocyanic acid and must neither interfere with the ferric thiocyanate reaction nor in the presence of mercuric thiocyanate

[22] W. W. Scott, "Standard Methods of Chemical Analysis," D. Van Nostrand Co., New York, 1925, 4 ed., p. 607.
[23] *J. Biol. Chem.,* **38**, 191 (1919).
[24] *J. Biol. Chem.,* **44**, 399 (1920); *J. Ind. Eng. Chem.,* **13**, 696 (1921).
[25] *J. Ind. Eng. Chem.,* **13**, 153 (1921).
[26] E. F. Burton, "The Physical Properties of Colloidal Solutions," Longmans, Green and Co., New York, 1921, 2 ed., p. 173.
[27] *J. Am. Chem. Soc.,* **29**, 409 (1907).
* See paper by R. Willstatter in this volume. *J. A.*

impart a color to amyl alcohol; or, if it does not meet these requirements, it must be capable of easy separation from the iron. Aluminum hydroxide would be the ideal collector were it not for the fact that it dissolves slowly and imperfectly in thiocyanic acid, and thus frequently prevents complete solution of the accompanying ferric hydroxide. Repeated experiments by Stokes and Cain showed that it is not to be depended on, and they have therefore employed it only in special cases where it was removed before final treatment of the precipitate with thiocyanic acid. The iron is precipiated either as sulfide or as ferric hydroxide. The hydroxide precipitation is employed in the absence of materials which have a solvent action such as citrates, tartrates, sugar and many other organic substances. pyrophosphates, arsenites, arsenates, antimonates, etc. The usual collector for ferric hydroxide is hydrated manganese peroxide. The sulfide precipitation is used when from the presence of any of the just mentioned substances, hydroxide would remain in solution. It is also used when other sulfides insoluble in ammonium or sodium sulfide are practically absent. The best collector for iron sulfide is cadmium sulfide. In this case the cadmium sulfide is redissolved and the iron reprecipitated as hydroxide with manganese dioxide as collector. In many cases the choice between the methods is optional. When there is reason to fear the presence of traces of organic matter, as in the case of materials which have been treated in wooden vessels in the process of manufacture, or when arsenic or other prejudicial substances may be present, as in the cruder reagents, the sulfide method is more accurate. For example, pure sodium chloride gave Stokes and Cain identical results by either method, while a sample of the best commercial chloride gave decidedly too low results with the hydroxide method.

Special care is necessary in sampling the substance, and wherever practicable duplicate determinations should be made on portions of the same solution, as it frequently happens that different samples, especially of crystallized substances, taken from the same bottle show widely varying results, owing to the irregular distribution of the iron.

Another illustration of the use of a "collector" is in the colorimetric determination of tungsten by the method of Travers.[28] This method is based upon the reduction of tungstic acid by titanium chloride, giving a blue colored oxide of tungsten that remains in colloidal suspension under certain conditions. If the sample is an alloy, it is treated directly with aqua regia; if a mineral, it is fused with sodium sulfite and the mass taken up with aqua regia. Most of the tungsten is precipitated as tungstic acid (along with the silica), but the separation is not quantitative due to the presence of metatungstic acid. The filtrate, which should contain iron (10% is sufficient), is treated with ammonium hydroxide until it is just alkaline to litmus. The ferric hydroxide thus precipitated entrains the tungstic acid and hence serves as a "collector." The precipitate is then washed free from sodium salts, dissolved on the filter in 6 N hydrochloric acid, and the solution added to the major part of the tungstic acid solution, after the latter has been freed from silica by any of the usual methods and taken up in hydrochloric acid. The combined solutions are evaporated to a volume of about 2 cc., cooled, diluted to 40 cc., 5 cc. of titanium chloride solution added, and the resulting blue colloidal solution diluted to 50 cc., mixed and compared at once with a standard similarly prepared.

[28] *Compt. rend.*, **166**, 416 (1918); cf. *ibid.*, **165**, 408 (1917).

Factors that may Influence the Size of Colloid Particles. In addition to the effect which stabilizers have on the size of colloid particles, there are such factors as the hydrogen ion concentration, concentration of electrolytes, temperature, light, nature of dispersion medium, etc. Any one or more of these may seriously influence the degree of dispersion of colloid particles, and hence the accuracy of the determination. It is, therefore, necessary to carry out all procedures (which deal with colloid suspensions) under carefully studied and well-defined conditions in order to attain the highest degree of precision and accuracy. Perhaps the most important precaution to take is to carry out the preparation of the unknown and standard under as nearly identical conditions as possible.

Folin and Wu, *J. Biol. Chem.*, **38**, 81 (1919).
Folin and Youngburg, *J. Biol. Chem.*, **38**, 111 (1919).
Youngburg, *J. Biol. Chem.*, **45**, 319 (1921).
Karr, *J. Lab. Clin. Med.*, **9**, 3 (1924).
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URIC ACID.

Folin and Wu, *J. Biol. Chem.*, **38**, 459 (1919).
Benedict, *J. Biol. Chem.*, **51**, 187 (1922).
Benedict and Franke, *J. Biol. Chem.*, **52**, 387 (1922).
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Newton and Dakin, *Science*, **64**, 602 (1926).
Benedict, Newton and Behre, *J. Biol. Chem.*, **67**, 267 (1926).

ZINC.

Birckner, *J. Biol. Chem.*, **38**, 191 (1919).
Bodansky, *J. Biol. Chem.*, **44**, 399 (1920).
Bodansky, *J. Ind. Eng. Chem.*, **13**, 696 (1921).

The Desirability of Sometimes Stating Reactions in Terms of Concentration Rather Than pH

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Because of its numerical simplicity and theoretical significance, Sørensen's pH plan of stating reaction has come into wide use in biological as well as chemical studies. The pH numbers are negative exponents,[1] and in any system where the phenomenon under consideration bears an exponential relation to the reaction of a liquid phase, these numbers are well suited for calculation, tabulation, and plotting. There exists, however, an unfortunate tendency to overlook the fact that biological phenomena may at times be related to hydrogen-ion *concentrations* instead of exponents, and that the use of pH numbers in such cases may lead to serious misinterpretation of the situation.

In a recent article[2] on a new method of stating hydrogen-ion concentration, two examples of such misinterpretation were pointed out. Another example has just come to hand, and as it deals with a fundamental biological problem, that of plant growth, it may well be discussed here in detail. Wann and Hopkins[3] prepared a complete growth-pH curve for *Chlorella* from the data of three separate experiments; it is reproduced in simplified form in Figure 1.

In describing this curve the authors state: "There appears to be no definite high point or maximum; instead the curve ascends rapidly from both the acid and alkaline limits to a region lying between a pH of 4.6 to a pH of 7.0, in which the rate of growth is quite uniform. Two suggestions might be offered to explain the "flat" portion of this curve. First, that the organism is not very sensitive to changes in H-ion concentration within this range, and second, that under the conditions of the experiment some other factor besides H-ion or OH-ion concentration is limiting between pH 4.6 and 7.0. . . . "at H-ion concentrations near pH 7.0 rather large amounts of

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[1] The following tabulation shows the relation of pH values to the hydrogen-ion concentrations:

H-ion Concentration, Grams per Liter	Abbreviation	pH Number
10	10^1	—1
1	10^0	0
0.1	10^-	1
0.01	10^-	2
0.001	10^-	3
0.0001	10^-	4
0.00001	10^-	5
0.000001	10^-	6
0.0000001	10^-	7
0.00000001	10^{-8}	8
and so on.		

In words, the pH numbers represent the *exponent of 10* corresponding to the hydrogen-ion concentration, expressed in grams per liter, *multiplied by —1.*

[2] Wherry, *Bull. Wagner Free Inst. Sci. Phila.*, **2**, 59 (1927); *Am. J. Pharm.*, **99**, 342 (1927).
[3] *Botanical Gaz.*, **83**, 194 (1927).
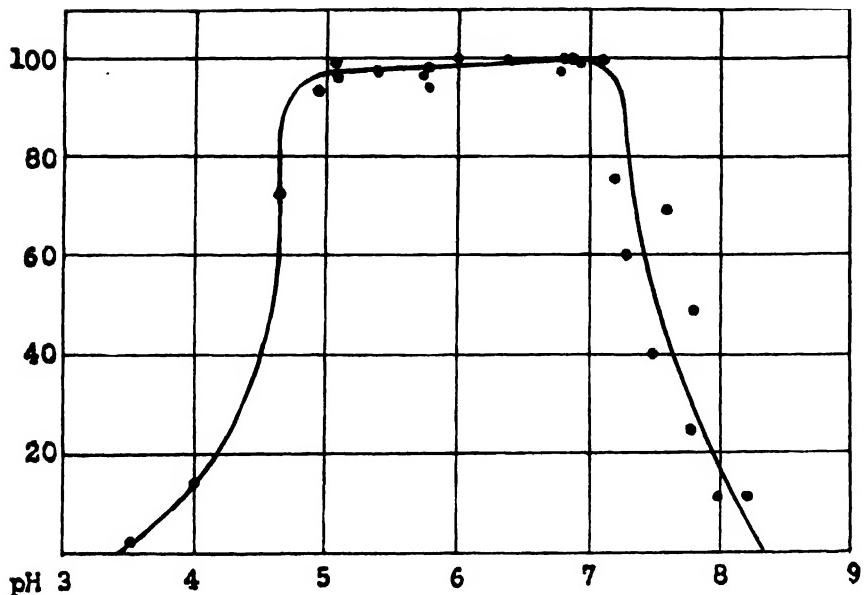
Fig. 1.—Relation between H ion exponents and growth of *Chlorella;* data from Wann and Hopkins.
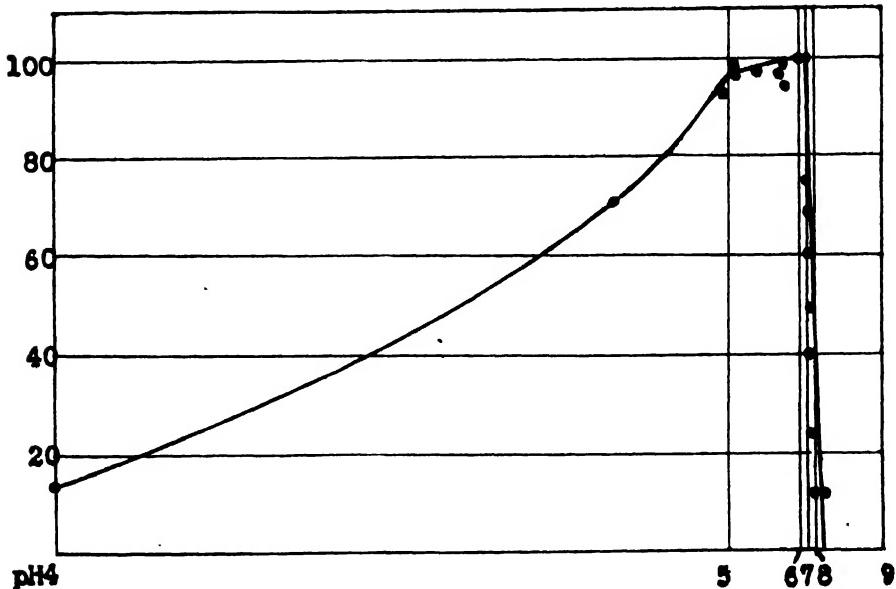
Fig. 2.

iron were necessary for maximum growth, amounts much greater than used in the experiments reported in this paper. It is possible, therefore, that by increasing the iron content in this region the flat part of the curve would disappear."

As a third suggestion concerning the flat portion of the curve I would call attention to the fact that the growth of *Chlorella* may well be related to the hydrogen and hydroxyl-ion concentration instead of to their exponents. The result of plotting the data of Wann and Hopkins on this basis is shown in Figure 2.

There are too few measurements of growth rate between pH 4 and 5 to be certain of the shape of the curve there, but in any case the flat portion is now so short as to require no special explanation, and there is a distinct maximum at pH 6-7. The feature which really calls for an explanation is the rapid drop in growth at pH numbers above 7, but the authors have supplied this in their remark that they did not furnish the plant with sufficient iron in the earlier experiments.

As the literature contains not a few other instances where the use of hydrogen-ion exponents (pH values) in plotting has obscured rather than clarified a biological problem, it may here be urged that in general curves should be drawn for the hydrogen-ion concentrations as well as for their exponents, and the significance of both kinds should be considered before reaching a final conclusion regarding the relations involved.

The usefulness of a concentration method is, moreover, not limited to the construction of graphs; such a method may render it possible to state reaction values in terms which the layman can understand. It is true that the old plan of using expressions like 5×10^{-6} is too difficult of comprehension, and that the antilogarithms of the pH numbers (first column in footnote 1) are too cumbersome for general use. There is, however, a relatively simple plan, apparently first suggested by Walker and Kay,[4] and gradually improved upon until it has attained the form given in my paper already cited.

The quantity of hydrogen-ion in a liter of pure water, 0.0000001 gram, is taken as a unit of "active acidity," and the same fraction of a gram equivalent of hydroxyl-ion is taken as a unit of "active alkalinity." From the quantity of either of these ions, thus expressed, is subtracted the quantity of the other (the two being reciprocals); the difference represents the excess of the respective ion which is free to take part in producing the reaction of the solution.[5]

This plan gives numbers which are not unduly large, and which can readily be evaluated by the layman. For instance, orange juice has an average pH value of 3.6; grape juice of 4.5. From these numbers few persons can tell offhand how much more acid one is than the other; but when the quantities are stated in active acidity terms, they become 2,500 and 316 respectively, and anyone can then see at a glance that the first liquid is 8 times as acid as the second. The rounded-off active acidity and active alkalinity equivalents of some frequently encountered pH values are as follows:

[4] *J. Soc. Chem. Ind.*, **31**, 1013 (1912).
[5] Giribaldo has proposed [*Biochem. Z.*, **163**, 9 (1925); *Bull. soc. chim. biol.*, **7**, 652 (1925)] to use the quotient H/OH instead of the difference H — OH or OH — H. This plan has the advantage of indicating acidity by a + sign and alkalinity by a — sign, but the numbers obtained have no real significance, and the curves obtained by plotting them against plant growth (or other phenomena) would be even less informing than those in which pH values are used.

pH No.	Active Acidity	pH No.	Active Acidity	pH No.	Active Alkalinity	pH No.	Active Alkalinity
4.0	1000	5.5	31.5	7.0	0.0	8.5	31.5
.1	800	.6	25	.1	0.5	.6	40
.2	630	.7	20	.2	1.0	.7	50
.3	500	.8	16	.3	1.5	.8	63
.4	400	.9	12.5	.4	2	.9	80
.5	315	6.0	10	.5	3	9.0	100
.6	250	.1	8	.6	4	.1	125
.7	200	.2	6	.7	5	.2	160
.8	160	.3	5	.8	6	.3	200
.9	120	.4	4	.9	8	.4	250
5.0	100	.5	3	8.0	10	.5	315
.1	80	.6	2	.1	12.5	.6	400
.2	63	.7	1.5	.2	16	.7	500
.3	50	.8	1.0	.3	20	.8	630
.4	40	.9	0 5	.4	25	.9	800

The statement of reaction in concentration rather than exponential terms may thus make it possible for scientists not only to present their data in a form more intelligible to the layman, but also to avoid drawing erroneous conclusions regarding the significance of their results. The desirability of following this plan more frequently than is customary at present, seems evident.

<div align="center">EDITOR'S NOTE.</div>

<div align="center">pII vs. cH.</div>

In a review of Dr. Jacques Loeb's "Proteins and the Theory of Colloidal Behavior," published in *Chemical and Metallurgical Engineering* in 1922, Jerome Alexander pointed out the danger of plotting pH instead of cII. These views, with some additions, were also expressed in a paper on "The Degree of Dispersion as an Influence in Tanning," published in *J. Am. Leather Chem. Assoc.*, August, 1923, as follows:

The expression pII demands some explanation. It is a convenient way to express the reaction of any fluid, but is dangerous because it does not do this directly, because it is an inverse logarithmic function, deprived of its minus sign, as you will see directly.

Pure water dissociates slightly according to the equation

$$H_2O \rightleftarrows H^+ + OH^-$$

the reaction being reversible. It has been found that at 22° C. the concentration of hydrogen ions in pure water equals $\dfrac{1}{10,000,000}$ moles per liter, a fact usually expressed by the symbolism cII⁺ (concentration of hydrogen ions) equals 10^{-7} moles per liter. Since for each H⁺ ion in water there is an OII⁻ ion, $cOH^- = 10^{-7}$ under the same conditions.

Now for convenience, Sörensen proposed to disregard the minus sign, and to use simply the numerical value of the exponent of 10 to express the reaction represented by the corresponding cII⁺. The following table will make this clear:

	Degree of normality	cH⁺	Equivalent pII value
HCl	1.0	8.0×10^{-1}	0.10
	0.1	8.4×10^{-2}	1.07
	0.01	9.5×10^{-3}	2.02
	0.001	9.7×10^{-4}	3.01
	0.0001	9.8×10^{-5}	4.01
Acetic acid	1.0	4.3×10^{-3}	2.37
	0.1	1.6×10^{-3}	2.87
	0.01	$4 3 \times 10^{-4}$	3.37
	0.001	1.6×10^{-4}	3 87

	Degree of normality	cH+	Equivalent pH value[*]
Caustic soda	1.0	0.90×10^{-14}	14.05
	0.1	0.86×10^{-13}	13.07
	0.01	0.76×10^{-12}	12.12
	0.001	0.74×10^{-11}	11.13

This table shows that the lower the pH value, the greater the *effective reaction* of acidity, which is something quite different from the *total* acidity. Thus equal volumes of normal hydrochloric acid and of normal acetic acid will neutralize equivalent volumes of normal alkali, but the normal acetic acid has a much lower degree of acidity or hydrogen ion concentration, and therefore a higher pH value.

Not only does the pH value run *opposite* the H ion concentration, but the variations are exponential or logarithmic, not arithmetical. The step between pH 5 and pH 6 is vastly less than the step between pH 5 and pH 4, as may be seen from the following table:

pH value	Number of time H (or OH) ion concentration exceeds that of pure water	
1	1,000,000	
2	100,000	
3	10,000	
4	1,000	acid side
5	100	
6	10	
7	0	pure water————————————
8	10	
9	100	
10	1,000	alkaline side
11	10,000	
12	100,000	
13	1,000,000	

The reaction of bio-colloids to minute changes in H ion concentration is remarkable. Thus fibrin will swell much n ore in ordinary distilled water than it will in the highly purified conductivity water, (pH — 7) because the absorption of carbonic acid from the air gives distilled water a pH of about 5 5. During life our circulating body fluids have a faint alkalinity, pH about 7.4, which apparently represents a condition of minimum swelling for the blood and most tissues. Very slight deviations from this either way produce alkalosis or acidosis, and speedy death. In fact upon death there is a sharp increase in the H ion concentration from about pH 7.4 (alkalinity) to about pH 6.4 (acidity).

To give some further striking idea as to what enormous gaps may be lightly jumped by a logarithmic notation, we may recall F. W. Aston's figures [*Nature*, 113, 393 (1924)] for the weights of the earth's divisions: Hydrosphere, 1 45 $\times$ 10²¹; lithosphere, 5.98 $\times$ 10²⁷; atmosphere, 5.29 $\times$ 10²¹ grams. That is, the exponential figures, corresponding to pH, would be about 24, 27, and 21, respectively. Putting it another way, the size of protons and of electrons is about 3×10^{-11} cm., and the diameter of the Milky Way (according to Shapleigh) is 10^{24} cm. (300,000 light-years), so that in an exponential spread of 37 we pass from the smallest known material units to the limits of the Galaxy.

they consist of a disperse internal or discontinuous phase and of an external or continuous phase. In contradistinction to gross suspensions, or emulsions, containing *microns,* that is, of particles visible with the microscope (Zsigmondy), suspensions of submicrons, that is, disperse systems whose particles are seen only with the ultramicroscope, e.g. hydrosols (suspensoids and emulsoids), are relatively stable.

In fact disperse systems are likewise solutions of crystalloids, because they too have a portion dispersed in the solvent as *amicrons,* that as ions or molecules. However, solutions (amicronic suspensions) are not heterogeneous systems, formed of several phases, in the sense of W. Gibbs.

With suspensions and colloid systems the "Formart" and the chemical nature of the disperse phase are not of much importance. A much more important fact is that while submicronic suspensions are heterogeneous or diphasic systems, solutions are homogeneous or monophasic.

The size of the molecules or ions of solutions, and of the disperse particles of submicronic suspensions, is of relatively little importance, provided the dimensions of the latter be within the conventional limits of from 1 to 100 $m\mu$. The molecules and the ions may be large, but if they do not form a phase distinct from the solvent, the system will always be a solution. The suspended particles may be extremely small; but if they form a distinct phase, we shall always have a heterogeneous system, which must be grouped with the submicronic suspensions, and not with solutions.

Is it possible to state that when the molecules or ions of a dissolved substance are large, they must for this reason alone form with the solvent in which they are dispersed a heterogeneous, diphasic system? It is well known that certain methods of dispersing metallic gold yield extremely minute particles, 2 to 6 $m\mu$ in diameter, but that nevertheless, colloidal gold is always considered as a heterogeneous system. If the minuteness of the disperse particles is not sufficient to transform a submicronic suspension into a solution, or an inhomogeneous system into a homogeneous one, we must also hold that if the molecules of a dissolved substance happen to be very large, this fact would not be a sufficient reason for considering the system diphasic.

This is the main point of the objection which I raised.

3. The fluid (aqueous) colloidal systems, or *hydrosols,* are divided into suspensoids and emulsoids, the former colloids whose particles are solids (colloidal metals, inorganic colloids). By the second term, however, we mean not only those heterogeneous systems whose disperse particles are fluid (i.e. a submicronic suspension of chloroform, or of benzol in water), but also those whose particles come from the submicronic dispersion in water of cooked starch, glycogen, denatured proteins, soaps of higher fatty acids, mucilages, etc. These substances, when pure, are not fluid. However, the systems formed by them are called emulsoids, because the disperse particles have a more or less strong affinity for water, and are therefore more or less hydrated; so that while we regard them as solid, inasmuch as they form a different phase from the dispersion medium, still we do not consider them hard or rigid, amorphous or crystalline, but more as having, so to say, a doughy consistency.

We know, however, as far as concerns the proteins especially, that their property of absorbing water, of hydrating, is very slight when they are near the isoelectric point. It is but little superior to that of suspensoids. Considering the protein hydrosols in such condition, there would therefore be no reason to separate them from other hydrosols.

We would, in a way, be induced to keep the distinction, in consideration of the fact that when the proteins are ionized, they can also become highly hydrated. But then the system, as will be seen later, will lose most of the characteristics of heterogeneous systems, and simply be regarded as a solution of ionized proteins, and no longer heterogeneous.

4. Under the name of hydrogels are usually grouped the solid systems arising, through precipitation or coagulation, from suspensoids and emulsoids.

Hydrosols are characterized by the presence in them of disperse particles: (1) separated from each other by a layer of the dispersion medium more or less thick but always uniformly distributed through the whole liquid mass; (2) showing continuous Brownian movement; (3) electrically charged, positively or negatively, but oppositely from the charge of the dispersion medium, so that if the hydrosol be submitted to the action of an electric field, the particles migrate to the anode or to the cathode (*electric cataphoresis*). Hydrogels, on the other hand, do not exhibit the last two characteristics, and as far as the first is concerned, they differ from hydrosols inasmuch as the disperse phase is no longer uniformly distributed, but massed in flocculi or lumps at the bottom or surface of the dispersing phase, where it forms a stratum richer in dispersed phase than in dispersion medium.

From hydrogels (gels) jellies (gelatin, gelée, Gallerte) are differentiated mainly by the following characteristics: (1) they are solid, compact systems, but with a certain elasticity that enables them to preserve their shape at least for a certain time; (2) in them, contrary to gels, there is no microscopic separation of a portion richer and one less rich of dispersing phase, because, during the gelatinization, the system, previously fluid, solidifies as a whole; (3) *although jellies are physically heterogeneous, polyphasic systems*, the fluid phase is not free from the solid phase, but it is imprisoned in it; (4) jellies may be opaque or translucent but gels are opaque as a rule; (5) the dispersed phase, when immobilized in jellies, cannot easily be redispersed; with gels it may as a rule be redispersed, though in certain cases only for a limited period following formation. Among solid, jellied colloidal systems are: coagulated blood plasma; coagulated egg albumin; sufficiently concentrated solutions of gelatin or of agar, or of soaps of high fatty acids prepared hot and then allowed to cool; coagulated milk, etc.

The mechanism of formation [1] is, however, in both cases the same. When a hydrosol coagulates or gelatinizes, there are formed groups of various sizes due to the aggregation of submicrons into microns. If the aggregates remain distinct, and as such, separate from the fluid, we have a gel or coagulum. If, instead, they unite in chains among themselves, forming a more or less dense reticulum, which retains in its meshes the liquid phase, we have a jelly. But in both coagulum and jelly, microscopic or ultramicroscopic examination usually reveals an inhomogeneous, polyphasic structure.

In some cases (gelatinization of the blood of certain animals, or of certain soap solutions) the submicronic aggregates are not amorphous granules, but assume a crystalline aspect. In other cases jellies have a reticulated or spongy structure, in still others it is alveolar; and, in these latter cases, according to the concentration of the solution, as for instance of gelatin, the internal phase (content of the alveolar cavity) may be solid, while the continuous phase is liquid; or, *vice versa*, the continuous phase (alveolar wall)

[1] R. Zsigmondy, "Kolloidchemie," 3rd ed., pp. 102, 365, *et seq.*, Leipzig, 1920.

may be solid, while the internal phase is liquid. This, however, has importance only insofar as in the latter instance, the liquid phase can be squeezed out or separated from the solid much less readily than in the former instance.

There are jellies which, however, "show no visible gel elements."[5] In fact, "concentrated jellies (6% and over) are so fine, that no differentiation is possible, even on intense solar illumination in the kardioid ultramicroscope. The existence of a fine amicroscopic discontinuity is shown by a distinct Tyndall phenomenon. The diffracted light is linear polarized." (Zsigmondy, *loc. cit.*, p. 367.)

In the less concentrated jellies (0.5-1%), "a distinct structure is visible in the ultramicroscope. Flocklike groups of submicrons are seen, which are quite distinguishable, and whose larger units generally exhibit an irregular shape."

Not always, therefore, do jellies exhibit a structure: not always are they formed of a simple solid frame-work containing in its meshes a solution of salted-out gelatin.[6]

We have, as a matter of fact, other examples of formation of a gelatinous system, optically homogeneous or nearly so, and solid or semisolid, formed through progressive concentration of a molecular disperse or granular disperse system. A dilute aqueous solution of sodium oleate is turbid; on hot concentration it slowly becomes clear, and on cooling it becomes a solid opalescent jelly. Similarly, on forcing the concentration of a hydrosol of glycogen, the turbid solution becomes clearer and clearer, and is finally transformed into a semisolid transparent jelly.[7] The reason these turbid systems clear when they solidify, is probably because the suspended particles of soap, glycogen, or gelatin, ultimately absorb all the aqueous phase, fusing together with a tendency to form a single phase, so that their turbidity continuously diminishes, and on ultramicroscopical examination, the number of submicrons becomes smaller.

It is also well known that the gelatinization of solutions of proteins, nucleins, blood serum, egg albumen, etc., of medium concentration, may be brought about by the action of concentrated alkali (also by an excess of acid); with more concentrated solutions of blood serum, simply by heating at 50 to 55° C.[8] In this instance it is not only the concentration of the colloid that produces the effect, but, above all, the extreme degree of ionization, and, therefore, of hydration, of the proteins.

"If the negative charge of albumin is first raised by action of alkali, it may take up a considerable amount of subsequently added water and nevertheless attain a high degree of internal friction. This rise in viscosity, under certain conditions (adding the dispersion medium in relatively small amount, so that the dispersed particles, so to say, compete for the water and interfere with each other's swelling— Pauli), leads in the case of alkali or acid sols of albumin, to jelly formation or gelatinization."[9]

Handovsky expresses similar views:[10] "The jelly state . . . is characterized by the fact that the disperse phase and the dispersion medium are intimately united with each other; from this closeness of union between the

[5] R. Zsigmondy, "Kolloidchemie," 3rd ed., pp. 365-367. Leipzig, 1920.
[6] D. J. Lloyd, *Bioch. J.*, 14, 147 and 584 (1920).
[7] Fil. Bottazzi and G. D'Errico, *Pfluger's Arch.*, 115, 359 (1910).
[8] See paper by M. Spiegel-Adolf in this volume. *J. A.*
[9] A. von Tschermak, "Allgemeine Physiologie," I, p. 152. Berlin, 1916-1924. *See also* pp. 67, 75, 84, 89, 90, 290, 291, 298, 299, 387, etc.
[10] H. Handovsky, "Der Kolloide Zustand," in C. Oppenheimer's Handb. d. Bioch., 2nd ed., Vol. 2, p. 59, Jena, 1923.

disperse phase and the dispersion medium in jellies, it immediately follows that the stability of form is maintained, with unorganized jellies if the fluid content is above 98 per cent, with organized jellies if the fluid content is even more; so that finally the disperse phase shows no Brownian movement. The jelly state is therefore an intermediate between solid and fluid." [11]

According to Mayer and Schaeffer,[12] jellies (the authors actually speak of *gels,* but evidently they refer to those systems now commonly spoken of as jellies) are "organic colloids" which are "optically almost homogeneous; that is, in the normal state present few, if any, granulations as distinct from the milieu"; and they mean to indicate both liquid systems ("plasma fluoré") and the protoplasm present between granules in the cells. "Typical gels (that is, jellies): silica, alcoholic solutions of soaps, pure collodion in alcohol-ether, albumin dialyzed against aqueous saline, plasma with fluoride (plasma fluoré), etc., *are absolutely devoid of visible granulations."* It is easily seen that for these authors the concept "jelly" implies the optical homogeneity, and does not exclude the liquid aggregation of the system, contrary to the opinion of the other authors just mentioned, who believe that a jelly is always a solid colloidal system, no matter how tender or almost doughy it might be, which can be optically empty or show on ultramicroscopic observation submicrons and aggregates of submicrons.

To conclude, the truth is that at present the term "jelly" is given to entirely different systems; e.g., to a bit of coagulated egg albumen or of gelatin, to a concentrated solution of collodion in ether-alcohol, to blood plasma, protoplasm, to an optically empty jelly or one which in the ultramicroscope shows numerous aggregates of submicrons, forming a reticular, spongy or alveolar structure, etc.; to a compact solid jelly from which no fluid exudes spontaneously, or to coagulated blood plasma, whose gelatinous consistency is due mainly to the fact that the fluid itself, almost unchanged, is caught in the meshes of a fine reticulum formed by the gelatinization of a minimal part of the total protein content, etc.

At any rate, what I wish to emphasize, because it is proved by many observations previously reported, is the existence of solid artificial colloidal systems, belonging to the jelly group, which being formed through an extreme concentration or hydration of particles previously disperse, with partial or

[11] The following parts of Handovsky's monograph deserve to be quoted: p. 60: "Jellies differ in their physical properties from other solid bodies (e.g. from soft or plastic substances), and also from liquids in view of their condition. Especially marked in jellies are the elasticity and stability of form characteristic of the solid state; they have, however, many properties of fluid substances."

P. 62: "The more water a jelly absorbs, the more it is dominated by fluidity; notwithstanding this jelly particles are not freely movable, but maintain their coherence. Taking the molecular weight of the gelatin 'molecule' as 10,000, a 1 per cent gelatin jelly has about 50,000 water molecules to each gelatin 'molecule.' In jellies there is marked movability of molecules with respect to each other, just as there is in fluids, but in addition there is a very high viscosity.

"Jellies are formed either by the setting of a sol, e.g. by allowing glue, starch, or soap *solutions to stand, or by swelling of the disperse phase in water. Every substance which forms a jelly has, therefore, the power of swelling,* but not in all fluids Thus, e.g, agar or gelatin swell in water but not in benzene, caoutchouc, on the other hand, swells in benzene but not in water. Certain relations therefore exist between the substance of the jelly and these fluids"

On page 97 Handovsky remarks that jellies of medium concentration "show in the slit ultramicroscope a faint bluish strongly polarized light-cone, which indicates that we are dealing not with a homogeneity, but with a very fine but definite heterogeneity; in observing this phenomenon, we see that *with increasing concentration the two phases become more like each other* (Wo. Ostwald) and that the diffraction discs of the individual particles are hidden by the closely packed dispersoid." He here referred to gelatin, that is to a denatured protein, and not in a favorable state of ionization to yield an optically homogeneous system.

Long before Handovsky and others, Mayer and Schaeffer had stated that "in gels (jelly), *colloidal granules, if they exist, are strongly bound to their solvent, they should be considered as swollen with the solvent, forming with it a homogeneous mass.* And, indeed, any action which diminishes the bond between colloid and solvent . will lead to the appearance in the gel (jelly) of ultramicroscopic granules, very fine at the outset, but becoming coarser and coarser."

[12] A. Mayer and G. Schaeffer, *Compt rend Soc Biol. d. Paris,* **64,** 681 (1908).

total disappearance of the continuous aqueous phase, and fusion or practical fusion of the greatly swollen particles, appear ultramicroscopically as homogeneous systems.

5. I stated that a colloidal system is usually considered as a polyphasic system.* Therefore, in it adsorption phenomena may occur. Some believe that the great importance of granular disperse systems is due to just these adsorption phenomena, which lead to concentration of one or more substances dissolved in the continuous phase, and therefore to the possibility of chemical reactions between the adsorbed substances being produced or accelerated by this local increase in concentration, of toxic substances dissolved in the disperse system being partly removed, etc. There is no doubt that metal hydrosols, inorganic and many organic sols, possess these fundamental characteristics of diphasic systems, the surface of the disperse phase increasing with the degree of dispersity, and that this is not characteristic of molecularly disperse or ionically disperse systems. The problem that we face is, therefore, the following: are we to consider as inhomogeneous systems the natural fluid proteins, such as blood plasma, pure lymph, egg albumen, etc., and at least the major part of solutions of artificial proteins in certain states, namely, when they strongly resemble in their physico-chemical characteristics colloidal physiological fluids?

There is no doubt that proteins, polysaccharids, lipoids, soaps of the higher fatty acids, are able to form diphasic systems similar to the common suspensoids or emulsoids, with disperse particles about 5 to 100 $m\mu$. Such would be a submicronic suspension of dialyzed blood globulin, of myosin, of myoprotein of dialyzed muscle juice, a dilute cold solution of gelatin (0.5-1%), a dialyzed solution of soap, etc. And, without any doubt, in these systems the particles in suspension are, as in the inorganic hydrosols, aggregations of molecules of proteins, soap, etc., without excluding the possibility that a larger or smaller proportion of the substance may be present as molecular dispersion. When proteins are in such condition, their particles are relatively poorly hydrated, and, similarly to the particles of the inorganic hydrosols, they are visible in the ultramicroscope, have little osmotic activity, do not greatly increase the viscosity, do not materially lower surface tension, are easily precipitated by inorganic ions and by colloidal particles of opposite electrical charge, are easily coagulated by heat, precipitated by alcohol, etc. If the proteins were always in this state, there would be no reason to differentiate their submicronic suspensions from other hydrosols; and, if we admit that the degree of dispersion of the substance and the inhomogeneity of the system characterize the colloidal state, protein hydrosols, those of soaps, of polysaccharids, etc., would form a united group with the inorganic suspensoids and emulsoids. But such is not the case.

Natural protein solutions (blood plasma, pure lymph, etc.) differ considerably from all artificial protein hydrosols; and from them differ, at least partially, artificial protein solutions, when they are under conditions similar to those of the physiological colloidal fluids. The disperse substance of the latter, contrary to that of the artificial hydrosols, behaves more like molecules and ions of solutions of crystalloids (in Graham's sense), than like suspensoid submicrons.

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* For a discussion of this point, see paper by E. Buchner in Vol. 1 of this series, entitled "Colloids as One-Phase Systems." *Also* R. Zsigmondy, "Colloids and the Ultramicroscope," Chapter I (New York, 1909). *J. A.*

Recently Wo. Ostwald [13] admitted that for certain colloidal systems and primarily for those of proteins, we are justified in speaking of "colloidal substances," because with them the power of forming colloidal particles is a chemical property, i.e. is inherent. Because of this characteristic he applied the term *eucolloids* to albumin, some lipoids, and some polysaccharids.

A similar idea is expressed by Pauli: [14]

"Under these conditions, there is no question of a sharp demarcation between the ordinary chemistry of compounds with large molecules (e.g. proteins) and colloid chemistry. The difference in these two cases is certainly not in the size of the particles, but in the domain of structure. When a metal or any simple compound is prepared in colloidal state, the dispersed particles always are made up of simple and similar sub-units. On the other hand, in non-associated compounds with large molecules, whose size is of the same order as the particles of these highly dispersed colloids, the disperse particles are closely connected and more or less heterogeneous atomic groups; thus, with albumin, the particles are composed of various amino acids, of whose distribution on the surface and in the interior of the molecule we have at present no knowledge whatever. Although at first glance the second case seems more complicated than that of a colloidal metal, we must not forget that the various chemical groups which are united in a substance with large molecules, often remain detectable by characteristic reactions, and impress their physico-chemical characters on the whole molecule."

But the problem does not limit itself merely to determining whether there are *colloidal substances*, "eucolloids," that is, substances capable of giving origin, because of their chemical nature or constitution, to systems having colloidal characteristics. More important is it to determine whether natural colloidal systems formed by eucolloids, are identical or not, with inorganic hydrosols, those of metals, etc.

I believe that they are not, for the following reasons:

Artificial dispersoids possess an osmotic pressure, a viscosity, an electrical conductivity, a surface tension, little different from that of the pure dispersion medium. The disperse phase carries positive or negative charges, but it is easily precipitated by addition of dilute solutions of salts, acids or alkali. The inhomogeneity of the system is easily revealed by ultramicroscopic observation; and if in some cases the particles are not clearly seen because of their tiny size, or the extreme concentration of the dispersoid, the inhomogeneity, the submicronic constitution of the system, is always revealed by a strong diffuse luminosity; and upon diluting the fluid, the luminosity resolves itself into bright points in Brownian movement. Furthermore, the particles of dispersoids cannot pass dialyzing membranes, nor through ultrafilters, and in them the disperse phase is very slightly hydrated. In these characteristics they differ greatly from molecular disperse systems, that is from the solutions of crystalloids, in the sense of Graham.

On the other hand, blood plasma has an osmotic pressure and a viscosity, due to proteins, greatly superior to that of the solvent (considering as such not water, but the solution of crystalloids that can be obtained from the plasma, for instance, by means of ultrafiltration). The colloidal substance is difficult to precipitate by simple neutralization of its electrical charge, which is always negative in neutral fluids, or by addition of dilute saline solutions. The fluid appears homogeneous in the ultramicroscope, with but a trace of diffuse luminosity; and dilution with the natural solvent, or even with physiological saline solution, does not produce the innumerable luminous submicrons

[13] Wo. Ostwald, "Kolloide und Ionen," *Kolloid Z.*, **32**, 1 (1923).
[14] Wo. Pauli, "Kolloidchemie der Eiweisskorper," Part I, p. 7. Dresden und Leipzig, 1920.

present in ordinary dispersoids. There may be more or less numerous luminous points or disks, but simple filtration of the plasma or serum will eliminate them; they usually are due to fragmentation of some cell, or to lack of cleanliness of the vessels containing the fluid. Finally, the colloidal material of the natural protein fluids appears strongly hydrated.

But the proteins of the plasma do not go through dialyzing membranes or ultrafilters. This, however, depends partly on the size of the protein molecules, and partly on the size of the pores of the membrane. Bechhold has demonstrated that with properly prepared membranes it is possible to make a fractional filtration of a mixed solution of various proteins; [15] and, at any rate, is this one characteristic sufficient to identify the two groups of systems, and to believe that natural protein fluids are also inhomogeneous, biphasic systems, in the sense of W. Gibbs? [16]

It must be kept in mind that we are able to prepare artificially submicronic suspensions of proteins that possess all the previously mentioned properties of submicronic suspensions of metals or of inorganic substances. But contrary to these, the former can easily be transformed into systems very similar, insofar as their physicochemical characteristics are concerned, to natural protein solutions. Take, for instance, an aqueous submicronic suspension of seroglobulin or very pure casein, or seralbumin dialyzed for several months. They exhibit osmotic pressure, surface tension, viscosity, electric conductivity, little different from that of the pure dispersion medium. Besides, the suspensions of casein or globulin are turbid; and the solution of seralbumin dialyzed for a very long time is opalescent. In the ultramicroscope, these fluids show many luminous submicrons. The protein contained in these fluids, if submitted to the action of an electric field, will show anodic cataphoresis; but the migration of the particles is extremely slow. Cations precipitate them readily, even in very small concentration. Submicronic suspensions of casein or of globulin are therefore heterogeneous biphasic systems. But if we add to them a small amount of sodium hydroxide, the fluid instantly becomes clear. Most of the luminous submicrons disappear; the osmotic pressure, the electrical conductivity and the viscosity increase. If the fluid is submitted to the action of an electric field, the protein migrates to the anode with much greater speed than shown before. Furthermore, the particles are highly hydrated, and not easily precipitated by addition of small quantities of salts, as before. What happens? The protein is transformed into sodium proteinate, which electrolytically dissociates and produces electronegative, strongly hydrated globulin ions. Probably the molecular groups of globulin forming the submicrons have split into smaller ones and in part into single molecules, and these have formed globulin ions, which being powerfully hydrated, no longer

[15] H. Bechhold, "Colloids in Biology and Medicine," tr by J. G. M. Bullowa.

[16] Very interesting are the ultramicroscopical pictures, recently described by Stübel [*Pfluger's Archiv.*, 156, 361 (1914)] and Howell [*Am. J. Physiol.*, 40, 526] of the spontaneous coagulation of the blood of mammals and higher crustaceans These authors observed that, during coagulation fibrin separates in the optically homogeneous plasma, according to the animal species, in form of granules or acicular crystals, or as filaments, numerous or extremely few (as in *Calappa*), forming a more or less compact network, always appearing brilliant, and containing serum in its meshes. Inasmuch as the content of the meshes appears ultramicroscopically homogeneous and not at all luminous, this proves that the serum remaining after the coagulation of the fibrinogen (which latter is but a very small portion of the total protein content) is a true solution of protein.

As we know, the blood of the higher crustaceans (*Homarus, Palinurus, Calappa*) when coagulated changes to a tremulous jelly, like animal gelatin; that is, to a system at once solid but soft; and the solid consistency is due to the fibrinous structure formed by coagulation of the fibrinogen. As in all jellies, then, even that of blood, merely one portion (with blood little, in other cases much) of the protein, is independently coagulated, and forms a reticulum, which contains in its meshes the balance of the uncoagulated protein.

differ materially in refractive index from the solvent, so that they will not be clearly seen in the ultramicroscope, although they are gigantic ions.

The question then is: Is this fluid an inhomogeneous diphasic system, and therefore similar to a metallic or inorganic dispersoid, or does it resemble more a homogeneous, monophasic molecular-disperse, or ion-disperse system, and therefore, a true crystalloid solution in the sense of Graham? I believe without doubt that the fluid has for the most part become a true solution of globulin; the granular disperse system has been transformed into a molecular-disperse and ion-disperse system, the heterogeneous system into a homogeneous, monophasic one.

It is true that the ultramicroscopic invisibility of the globulin ions is not significant. A suspension of very fine particles of glass in Canada balsam of the same refractive index cannot be optically differentiated into two phases, although no one could say that the glass particles do not form a phase distinct from the balsam. But true protein solutions differ from metallic and inorganic dispersoids, and resemble molecular-disperse and ion-disperse systems, not only because their particles are invisible in the ultramicroscope, but also because of all the other physico-chemical properties before mentioned. We must therefore give more weight to the osmotic pressure, to the viscosity, to the electrical conductivity of the fluids—in short, to the hydration of the disperse substance. Judging impartially, we must admit with Hoeber [17] that ultramicroscopically, the difference evident between suspensoid and protein solutions is that the latter usually contain "only few and very luminous submicrons" (Hoeber) in a diffuse, slightly luminous field. A qualitative difference, as far as the diffusion through membranes is concerned, does not exist between protein solutions and crystalloids.[18]

To differentiate between monophasic and biphasic systems, it seems to me that we should take as a criterion the relationship between the disperse substance and the dispersion medium. In diphasic systems there is an abrupt passage from one phase to the other—figuratively speaking, a jump between phases. In monophasic systems such a jump does not exist. The absence of such jump, the gradual passage from solvent to disperse particles, or *vice versa*, is also true in case of very large particles, e.g. protein molecules, provided they are highly hydrated and this maximal hydration is obtained exactly by the protein ions.*

An even more reliable criterion could be based on adsorption phenomena. If a true protein solution is not biphasic, if the molecules and ions do not form a separate phase in the system, no adsorption of free substances by the protein molecules and ions will occur in it; but if it is a biphasic heterogeneous system, the disperse phase must adsorb substances dissolved in the dispersion medium. Experimentally, however, it is not an easy problem. Freundlich [19] admits that there are no reliable data on this effect, and evidently they do

[17] R. Hober, "Physikalische Chemie der Zelle und der Gewebe," 5th ed., Part I, Leipzig, 1922.
[18] Höber says (*loc. cit.*, p. 289): ". . . not only is a certain amount of free diffusibility present in colloids, but also a certain degree of membrane diffusibility. The velocity of this membrane diffusibility is generally smaller the richer the membrane is in colloids, and the poorer it is in water. *This does not constitute a qualitative difference as against the membrane diffusibility of crystalloids.* [Thomas Graham emphasized the fact that colloids diffuse, and that the difference between colloids and crystalloids in this respect is *quantitative* only. *J. A.*]
  Tschermak (*loc. cit.*, p. 78, note 8) also says: "Thus undenatured protein solutions show relatively few submicrons (denatured ones very many!), but indicate the presence of colloidal (?) and even of truly dissolved amicrons."
  * Cf. the views of Gouy, regarding an "atmosphere about ions" (Vol. I). See also paper by Wo. Pauli, this volume. *J. A.*
[19] H. Freundlich, "Kapillarchemie," 2nd ed., p. 739. Leipzig, 1922.

not exist because investigators have not experimented along this line, being contented to assume, without experimental data, that all protein fluids are biphasic, heterogeneous systems. Freundlich quotes some experimental work to this end, but these experiments do not satisfy elementary requirements. In fact, owing to the nature of the adsorbing substance, it is necessary in such experiments to use substances that will not form chemical combinations with the proteins, nor have a tendency to produce aggregation of its particles, let alone precipitate them.

It is also absolutely necessary that the substance to be adsorbed should be molecular-disperse in the solution, because, if it were present as submicronic particles, first, it would have little or no tendency to concentrate at the interface, and second, it would itself act as an adsorbing agent, thus vitiating the experiment completely.

6. There is some disagreement among investigators as to whether proteins in solution are molecular-disperse, or are present as molecular aggregations, that is, as submicrons. This point is of extreme importance in my thesis, because it is only when physiological fluids are molecular and ionic solutions that we can consider them to be homogeneous systems. According to the recent work of J. Loeb [20] the possibility of a molecular dispersion of the proteins should be admitted. On the other hand, Bayliss [21] believes that proteins, when in solution, are always present as molecular aggregations, aggregations of hundreds and thousands of molecules. I believe, instead, that, especially in physiological solutions, the proteins are, at least in part, present in molecular and ionic dispersion; which does not exclude part of them being present as disperse molecular aggregations; as a matter of fact there is probably an equilibrium between the two.[22]

Another support for my thesis is furnished by the results of determinations of the surface tension which I made on solutions of sodium oleate,[23] of hemoglobin [24] and in collaboration with my assistant Dr. d'Agostino [25] on various proteins, by Quagliariello [26] on hemoglobin, and finally by me in collaboration with de Caro [27] on seralbumin and gelatin. We have observed that

[20] J. Loeb, "Proteins and the theory of colloidal behavior," New York, 1922.

[21] W. M. Bayliss, "The colloidal state in its medical and physiological aspects," pp. 30 and 85, London, 1923. *Idem.*, "Interfacial forces and phenomena in physiology," London, 1923. Here the author is still more explicit. In fact, he says (p. 74): "the protein ions like other large organic ions must be always aggregated. Thus we are led to the conclusion that proteins exist as aggregates not only at the isoelectric point, but even when in the form of salts. This being so, they always have surface properties." But these ideas of Bayliss are not shared by various other authors.

[22] H. Handovsky (*loc. cit.*, p. 48), says, in fact: "Colloid systems are basically heterodisperse. The heterodispersity may so change that the disperse phase is both colloidally and molecularly dispersed, as is the case, e.g. with dyes."
   I believe that proteins, more than any other substance, possess the property of passing from the molecular and ionic state, to that of submicronic aggregations, and vice versa. The prevailing numerical relation in various conditions of ions, or of the non-dissociated molecules, or of the molecules, or of molecular aggregations in form of submicrons, depends mainly on the reaction, presence of electrolytes, amount of solvent, temperature, etc.

[23] Fil. Bottazzi, "Sulla tensione superficiale delle soluzioni e sospensioni di saponi," *Rend. accad. Lincei* (5), 21, 365 (1912).

[24] Fil. Bottazzi, "Sopra alcune proprietà colloidali della emoglobina. II Modificazioni della viscosità e della tensione superficiale di sospensioni di metemoglobina per l'azione di HCl e di NaOH." *Rend. accad. Lincei* (5), 22 (2° sem.), 263 (1913).

[25] Fil. Bottazzi, "Sulla tensione superficiale delle soluzioni proteiche. I." *Rend. accad. Lincei* (5), 21 (2° sem.), 221 (1912). *Idem.* e d'Agostino, "Sulla tensione superficia le delle soluzioni proteiche. II," *Ibidem*, p. 561. *Idem.* e *Idem.* "Viscosità e tensione superficiale di sospensioni e soluzioni di proteine muscolari sotto l'influenza di acidi e di basi," *Ibidem*, 22 (2° sem.), 183 (1913).

[26] G. Quagliariello, "Influenza degli acidi e degli alkali su akune proprietà chimico-fisiche dell' emoglobina," *Arch. Sc. Biol.*, 2, 423 (1921). *Idem.*, "Azione degli acidi e degli alcali sull'emoglobina," *Ibidem.*, 3, 436 (1922).

[27] Unpublished researches. The results were communicated to the International Physiological Congress in Edinburgh (July 23-27, 1923).
   From the work referred to and from other experiments previously conducted by my assistant G. Buglia (*Biochem. Z.*, 11, 1908), we arrive at the conclusion that the surface tension of blood plasma is not the minimum possible, as far as its proteins are concerned, because they are in great part present

the proteins lower the surface tension especially when they are at the isoelectric point, while the surface tension of the solutions increases when they are ionized by addition of acids or bases, in proportion to the ionization.

On the other hand, submicronic suspensions of the various proteins previously mentioned, like those of inorganic suspensoids, have a surface tension approximating that of water. It is, therefore, the protein in the molecular state, undissociated, which, being relatively less hydrophile than the protein ions, lowers the surface tension; and not the molecular aggregations forming the submicrons which act as inorganic particles, nor the protein ions, which are always greatly hydrated. It is easily understood that more convincing results are obtained with proteins that give stable solutions at the isoelectric point, like seralbumin, oxyhemoglobin, gelatin, than with the proteins which at the isoelectric point are very unstable and precipitate, like seroglobulin and myoprotein.

7. Let us now consider protoplasm and the firm colloidal systems of the living organism. It would be absurd to consider protoplasm as a hydrogel, if by hydrogel we mean a system that has the physical characteristics of a precipitate, or a coagulum. Nor can protoplasm be considered as a simple protein solution, similar for instance to blood plasma. It is surely a fluid system, but has a consistency (as a matter of fact very variable) usually much higher than that of blood plasma. It could be considered, from the physicochemical standpoint, as concentrated blood plasma, in view of its colloidal components, although the consistency of the cell must be considered as due in part to its differentiated structures.*

I wish, however, to eliminate a misunderstanding that may be induced by my statement. I believe that *protoplasm* should be differentiated from *cytoplasm*. By cytoplasm, I mean the polyphasic system, complex and heterogeneous, formed of coexisting phases (Zwaardemaker) in which the liquid phase is the *undifferentiated protoplasm,* the other phases being the permanent structural differentiations, granular, bacillary, fibrillar, etc. (*differentiated protoplasm*), and the various inclusions. Now, undifferentiated protoplasm must exist to some extent in all cells, and originally it is probably the generator of all the protoplasmic differentiations. If it is one of the phases, it is homogeneous, it is the living matter from which are generated and regenerated the various endogenous granulations visible in the cytoplasm with the microscope or ultramicroscope, and the various structural differentiations. There must be, in fact, a medium in which the endogenous granulations, the fibrillae, the exogenous inclusions are imbedded; this medium is the protoplasm. During intense secretive activity, glandular cells excrete most of their granulations; then, during the following rest, these are regenerated. Very likely the micronic granulations are generated by submicronic particles, and these by amicronic particles which form the fundamental homogeneous protoplasm. We must admit that this protoplasm is quite able to form very minute granules in a reversible manner. That is, as the granulations form from the homogeneous protoplasm by the aggregation of amicrons, in like manner they disaggregate on returning to amicrons of the homogeneous proto-

as electronegative protein ions; but the minimum is reached if acid is added in amount sufficient to bring the proteins to the isoelectric point.

It is very likely that the meiostagmin reaction of M. Ascoli and G. Izar is due, at least partly, to a diminution in the degree of ionization of the proteins of the blood plasma. (M. Ascoli, *Münch. med. Woch.*, 1910; M. Ascoli and G Izar. *ibid ,* Nos. 4, 8, 22, 41; G. Izar, *Biochem. Z..* 29, 13 (1910) *Ber. Klin. Woch.,* 1911, No. 39. M. Ascoli, *Ergebn. inner. Medizin Kinderheilk,* 25, 944 (1924).

* On protoplasmic structure, see also several independent papers in this volume. *J. A.*

plasm, from which they originate. In other words, protoplasm can change reversibly from a homogeneous system into a heterogeneous one.

Probably physiological stimuli (and death) usually produce such changes,[28] because the acids formed in cells during stimulation bring the protoplasmatic proteins temporarily to the isoelectric point, for, in fact, they are already quite near to it when at rest. Later on, the system, having become heterogeneous or diphasic during the period of stimulation, returns to the homogeneous state in the recovery period. If for any reason the protoplasm does not come back to the homogeneous state, it is dead. The dead protoplasm is a permanently heterogeneous colloidal system. Probably, in physiological conditions, a true granularity is never reached, and the modification induced by acids is probably limited to a diminution of the degree of ionization of the proteins, that is, to a transformation of the protein ions into undissociated protein molecules having a distinct tendency to aggregate, or become granular.

The possibility, however, that a process similar to gelatinization might occur in highly stimulated protoplasm, and that in the initial stage such process might still be reversible, has been demonstrated by recent observations of Bayliss,[29] according to whom the Brownian movements cease within electrically stimulated cells, but return as soon as the stimulation is discontinued.

If protoplasmatic proteins, under the action of an excess of acid or alkali, become exceedingly stable either in the acid or in the alkaline region, the protoplasm thereupon must be considered as dead. The relative instability conferred on the cellular proteins by proximity to the isoelectric point, and the presence of salts in the cellular fluid, especially of bivalent cations, makes vital phenomena possible. To permanent stability there corresponds instead, lack of excitability.

The approximation of protoplasmatic proteins to the isoelectric point, and perhaps their reaching it, is a phenomenon associated with the specific functional activity of the protoplasm, which usually is intermittent or periodic (muscular contraction, glandular secretion, propagation of the stimulus along the neuron, etc.). Besides, the capacity to generate and regenerate permanent micronic and submicronic granules has probably a fundamental importance for the general metabolic activity of the cell, because, as the *cytoplasm* becomes thus a polyphasic heterogeneous system, phenomena of adsorption may occur in it on a large scale. At present, there is a tendency to accept the view that, as was mentioned above, the acceleration of chemical reactions in granular disperse systems is mainly due to adsorptive concentration of the reacting substances at the interfaces of the various phases, and to a specific superficial orientation of their more actively reacting atomic groups. (Langmuir.) As cells contain micronic and submicronic granules (zymogenous granules, condriomes), besides the permanent, functionally specific, structural differentiations (that is, they show the constitution of heterogeneous systems), it is possible that the reactive substances may concentrate upon the disperse phase, and the reaction become accelerated. A cell highly active functionally, is

[28] R. S. Lillie, "Protoplasmic action and nervous action," p. 59 (Chicago, 1923), writes: "I was struck with the constancy and definiteness of the relations existing between changes of translucency and changes of contractile activity (of the ctenophore swimming plate), which offers a typical example of translucent protoplasm. In dying animals the plates become partially clouded . . ." And on page 64: "The most obvious general structural changes occurring in the cell interior at death are of a coagulative kind; the protoplasm loses its normal translucency and becomes more opaque (death rigor or death coagulation)."

[29] The phenomena observed by Bayliss may be due, therefore, not only to gelatinization, that is, to coagulation of the colloidal protoplasm due to its being brought to the isoelectric point by acids formed in the cell, but also to a jellification of the protoplasm, caused by excessive acid formation which leads to hydration of the colloid.

always rich in granulations. However, it must not be forgotten that the *protoplasm* that generates the granules must surely be considered a *liquid homogeneous phase*, whose main chemical constituents are specific proteins, lipoids, mineral salts, etc., besides water. The variable viscosity [30] of the cytoplasm is due not only to its lipoprotein constitution, with its always highly concentrated, more or less ionized and therefore much hydrated colloidal constituents, but also to the number and nature of the granules and of the other structural formations, which are suspended therein.

If protoplasm is a very concentrated homogeneous colloidal liquid, it must also be optically homogeneous. This statement may sound like heresy, but only to those not having in mind the distinction which I made between *protoplasm* and *cytoplasm*. On ultramicroscopic examination, every cytoplasm shows glittering points and disks, because every cytoplasm contains granules, droplets and other disperse materials; in other words, *every cytoplasm is a suspension-emulsion*. Inasmuch as I call cytoplasm what everybody else indicates as protoplasm, there is no conflict between my statement and the definitions of "protoplasm" (according to my nomenclature "cytoplasm") given by Lillie (*loc. cit.* p. 67: ". . . the general or fundamental structure of protoplasm corresponds more closely to that of an emulsion than to that of any other simple non-living physical system"), Tschermak (*loc. cit. passim*), and others.

But the protoplasm, itself one of the phases of this polyphasic system, that is, the continuous or external phase, must, according to definition, be homogeneous.[31] If in an aqueous solution of saccharose we emulsify oil or liquid paraffin, and add powdered talcum, or charcoal, etc., we obtain a polyphasic system, of which the solution of saccharose is the continuous or external phase, which cannot be but homogeneous. To this homogeneous phase corresponds protoplasm; to the whole polyphasic system, the cytoplasm, the cell.

[30] Fr. Weber, "Die Viskosität des Protoplasmas" *Naturwiss. Woch. N. F.* 21

[31] H. Handovsky ("Der Kolloide Zustand," p. 98) says: "Living animal and plant protoplasm (*here read cytoplasm*) show a similar ultramicroscopic picture; they often have fine particles with Brownian motion, and larger ones without it; *the protoplasm intermediate* between them, is optically uniform."

Similar views are expressed by M. R. Lewis ["Reversible gelation in living cells," *Bull. Johns Hopkins Hospital* 34, No. 393, p. 373 (1923)]. "In the cells in tissue cultures, examined by W. H. Lewis ["Observations on cells in tissue-cultures with dark field illumination," *Anat. Rec.*, 26, 15 (1923)], there were no minute particles other than the mitochondria, neutral-red granules and fat droplets, visible in these cells even under intense dark field illumination with a Zeiss baby arc, except under abnormal conditions, when very small white granules (death granules) appeared, both in the nucleus and in the cytoplasm. These granules exhibit Brownian movement only when in the cytoplasm, never in the nucleus. Why no particles are visible in the protoplasm of cells in tissue-cultures is difficult to surmise. It may be that under normal conditions the particles are so small that they can be seen only after a certain amount of clotting has taken place, as Mathews (1920) describes for casein solutions. Or it may be that since these cells are spread out with so much more thinness than can possibly be obtained with living cells by any other means, the particles may be so arranged that the light is not reflected from them."

A number of years ago, A. Mayer and G. Schaeffer ["Sur la structure des gels. Application à l'étude de la constitution du protoplasma et des liquides de l'organisme," *Compt. rend. Soc. Biol. Paris,* 64, 682 (1908)] had written "Animal protoplasm when examined in living condition . . . often contains a certain number of microscopic granulations. These granulations, we may say, are never subject to Brownian motion. *When the granulations are few, between them the protoplasm appears optically homogeneous.* It appears simply like a jelly, like a gel." (*Vide supra* as to the significance of these terms.) "We know, in a general way, that protoplasm has a feeble alkaline reaction. Now, (1) like all alkaline or negative gels, they cloud up when penetrated by acids; that is, there appear grains at first ultramicroscopic, then microscopic. On the other hand, they become homogeneous when alkaline. (2) Acids, salts of heavy metals, and in general all substances employed as fixatives (there are no alkaline fixatives), act on protoplasm as on any negative gel, causing the appearance of grains which precipitate. Dehydrators (heat, alcohol) act the same way. There is reason to believe that the fundamental structures that have been described in protoplasm (grains, network, etc.), do not exist at all in the living state."

"Gaidukov has said that protoplasm is a sol, that life is characterized by the Brownian motion of its granulations, and that the stoppage of these movements (under the action of lethal agents) is the sign of death. He has examined highly aqueous *vegetable* protoplasms, in which the granulations are agitated by movements. We believe that the liquid which contains them, is in reality a homogeneous very fluid gel. Lethal agents, in coagulating it, hold the granulations imprisoned motionless."

To prove that protoplasm is optically heterogeneous the ultramicroscopical observations of Gaidukov [32] on vegetable cells, and of Mott [33] and Marinesco [34] on neurons are often quoted. But it is sufficient to examine the pictures (i) of these authors to become convinced that the brilliant granules observed by them are the ordinary ones seen in every cell, and other submicrons merely made visible by the paraboloid condenser. There is, however, between the brilliant granules, a non-brilliant homogeneous phase, which exhibits a more or less strong diffuse luminosity in which granules cannot be recognized; *that* is the protoplasm. This phase is optically homogeneous, and it is relatively more abundant when the cytoplasm is less differentiated and granular.[35] This optically homogeneous phase closely resembles the blood plasma of mammalia, or the blood of cephalopoda and crustacea. These fluids closely observed in the ultramicroscope, after separation of the morphological elements and repeated filtration, are optically empty, or at the most show a more or less scanty diffuse luminosity, in which, however, submicrons cannot be recognized, no matter how much they are diluted with fluids incapable of precipitating their proteins.* When the diffuse luminosity is present, as in the blood of *Palinurus,* it simply means that we are dealing with disperse particles which, although they are protein molecules and ions of very large size, yet do not make a distinct solid or liquid phase.

In the protoplasm (in the protoplasmatic phase of the polyphasic system which constitutes cytoplasm) we differentiate with Seifriz [36] two parts: the internal, which has less consistency, and the cortical that is more consistent. As far as the viscosity is concerned, the former resembles concentrated glycerin, or paraffin oil cooled to about 20° C.; the latter has the consistency of vaseline, of a mixture of plastic paraffins, of bread-dough; and it has considerable elasticity. But neither can be considered as a "gel" or "jelly,"

[32] N. Gaidukov, "Dunkelfeldbeleuchtung und Ultramikroskopie in der Biologie und in der Medizin," Jena, 1910.

[33] Bayliss, *loc. cit.* (21).

[34] G. Marinesco, "Étude ultramicroscopique des cellules des ganglions des animaux nouveau-nés," *Copt. rend. Soc. Biol.,* 70, 1057 (1911). *Idem.,* "L'importance des phénomènes physico-chimiques dans le mécanisme de certains phénomènes de la vie des cellules des centres nerveux," Volume publié en souvenir de Louis Oliver, Paris, 1911. *Idem.* et J. Minea, "Étude des cellules des ganglions spinaux de grenouille a l'aide du paraboloide de Zeiss," *Compt. rend. Soc. Biol.,* 71, 202 (1911).

[35] The authors who describe the protoplasm (cytoplasm) as a complex heterogeneous system of coexistent phases, never clearly state which is the continuous or external phase of such system, the one that holds in suspension the other phases, solid or liquid. Lillie (*loc. cit.,* p. 217). "The permanent structural elements are chiefly protein in composition, probably associated with lipoid; and this fact favors the inference that the interfaces between the solid protein structures of the cell and the adjoining more fluid phases are the site of the biologically essential reactions, etc." What are these "adjoining more fluid phases"? If they also are internal phases, there must of necessity be a liquid phase in which they are dispersed, along with the "solid protein structures."

Also Tschermak (*loc. cit.,* p. 396) spoke of and greatly emphasized this external phase. "Exactly in the sense of the new conception of the plasma as a system of co-existing phases . . . we must always be on guard against minimizing the continuous phase or phase complex, and giving weight principally or even entirely to the disperse phases or phase complex." I do not understand why he speaks of plural "continuous phases" because there can be but one for each polyphasic system. But that is how he expresses himself when giving a definition of this continuous phase

"As to a mode of dispersion which may underlie interchange in time and space, it is most probable that in the interior of the cell we have *dispersion in water* as the dispersion medium, whereas in the cell membrane and in special intracellular structures, there may be a distribution into fats or lipoids."

According to Tschermak, therefore, we may have in the same cell two continuous phases, water inside, fats or lipoids on the membrane, etc. Such a view not only sounds absurd to me, but is in contradiction with the importance given by the author to the "zusammenhaengende phase" in contrast with the "dispersen phasen" of the cytoplasm. What biological importance could be given a continuous phase of water"? In emphasizing the biological importance of this "zusammenhaengende Phase" we must admit that it possesses a protoplasmatic nature, made up as previously stated, of undifferentiated, homogeneous protoplasm, which is part of every cell, even of the most differentiated.

* It is a question as to how great an extent we have here an example of colloidal protection, or cumulative protection. See Vol. 1, this series. *J. A.*

[36] W. Seifriz, "The structure of protoplasm and of inorganic gels: an analogy," *Brit. J. Exper. Biology,* 1, 431 (1924). According to this author, one may, in general, reject the distinction between "protoplasm" and "cytoplasm," and the impossibility of considering protoplasm as a gel.

because these systems possess a structure, and contrary to protoplasm, are optically not homogeneous. And, as Seifriz reports, if the cytoplasm is submitted to mechanical action, it collapses, like a gel of iron hydroxide or cadmium; which is due probably to the fact that under the influence of the mechanical insults the protoplasm colloid becomes granular, loses water and becomes a hydrosol.

It is not necessary to assume that protoplasm has a structure in order to explain the consistency, even of its cortical portion, and its variations in viscosity. A homogeneous liquid like glycerin or liquid paraffin may show great variations in consistency and viscosity, due to variations of concentration and temperature.

8. There are also, in organisms, normal firm formations which, considered superficially, resemble jellies; soft formations almost of the consistency of coagulated blood of crustaceans, e.g. the crystalline lens; firmer still, are the cellulose walls of vegetable cells, hyaline cartilage, the fibrils of elastic and connective fibrils and membranes, cilia, chitinous cuticle, etc. Now, how should these formations be considered from the standpoint of colloid chemistry? Have they a structure similar to that of ordinary jellies? Are they solid, optically homogeneous systems, or heterogeneous? Are they polyphasic, or monophasic solid systems in the same sense that we consider perfectly transparent crystals? On this subject there are no investigations worthy of notice. But it seems to me that, for instance, a fibre of the crystalline lens, the fundamental substance of hyaline cartilage, etc., like ordinary jellies, are not to be considered as biphasic systems, formed of a solid phase (granular, filamentous, or reticular, etc.) and of a liquid one immobilized amongst the granules, and the granular conglomerations united by bridges in turn also granular.

We are rather induced to believe that they resemble crystalline formations, that is, homogeneous solid systems, and that they originate through concentration, or through concentration and hydration (vide supra) of the protein material of which they are formed, with peculiar orientations of the micelle, during organic development. Such a process of concentration, which takes place during histological differentiation, could very well have as its effect a complete coalescence of the primitive protoplasmatic particles, as it occurs under the influence of pressures and tensions, following definite lines, and could lead to the formation, for instance, of that solid transparent system, so well adapted to the optic function, namely, the crystalline lens; or in other cases to the formation of connective and elastic fibrils, of the starch grains of seeds, of the cellulose walls of plant-cells, etc., that is, to formations in which we may discover, with the aid of Roentgen rays, a crystalline metastructure.*

The researches on the behavior of organic structures to the X-ray examination have just begun; as a matter of fact I know only of the work of Herzog and Jancke. But this [37] already shows that the silk filaments of the silk-worm and of the spider, also human hair, when examined by X-rays, present a crystal-like metastructure.

* Regarding curious facts developed by ultramicroscopic examination of the vitreous humor, see Vol. 1 of this series, p. 818, paper by Prof. R. Zsigmondy. *J. A.*
[37] R. O. Herzog and W. Jancke, "Verwendung von Rontgenstrahlen zur Untersuchung metamikroscopischer biologischer Strukturen," *Festschr. Kaiser Wilhelm Gesellschaft zur Forderung der Wiss.*, p. 118, Berlin, 1921. *See also* W. H. Bragg and W. L. Bragg, "X-rays and crystal- structure," 4th ed., London, 1924. *See particularly* p. 134: "In many cases, substances thought to be amorphous have proved to be crystalline in nature. Even such substances as cellulose show a regular pattern when submitted to this method of analysis. A. Stock, "Ultrastrukturchemie, Ein leichtverstandlicher Bericht," Berlin, 1920. R. Zsigmondy, *loc. cit.*, pp. 112-113.

Scherrer,[38] in 1918, with similar researches, has proved that while suspensoids of gold or silver appear formed of very minute crystalline fragments, the hydrosols of gelatin and albumin on the other hand appear amorphous. Such constitution of artificial protein systems, however, does not mean that the natural protoplasmatic micellæ may not assume crystalline structure. Myofibrils without doubt are crystalline structures, and yet, in muscle juice, the submicronic fragments, to which they have been reduced, no longer show birefringence (unpublished researches of Quagliariello). But the observations of Scherrer could also be cited to support my view, *i.e.*, that hydrogels, just like protein hydrosols, are amorphous heterogeneous systems, and therefore differ profoundly from natural solid colloidal systems.[39]

9. In conclusion, among the solids and fluid colloidal systems of the living organism, above mentioned, blood plasma and the red blood cells of mammals are unquestionably homogeneous systems, and probably also the heads of spermatozoa, the resting cell nucleus, the crystalline lens, etc. The cytoplasm, on the other hand, is a polyphasic system, and therefore heterogeneous; but its continuous phase, protoplasm, is also necessarily homogeneous, whether it be present in small or large amount in relation to other phases. The polyphasic structure of the cytoplasm is highly favorable to the chemical processes of metabolism that continuously take place in it, because it makes possible, to a high degree, the phenomena of adsorption of reacting substances. And probably it is opportune that the protoplasmatic colloid, which forms the continuous phase of every cell, is near the isoelectric point, and is, therefore, in condition to be easily brought to the isoelectric point by the acids that form during excitation, in a reversible manner, because this reversible process is probably associated with changes in permeability which accompany functional activity and the return of the excited cells to the condition of rest.

It seems opportune to me to point out that, if protoplasm forms the continuous phase of the cells, it is not correct to accept the mosaic structure of the so-called "cellular membrane," that is, the formation of the membrane by the close fitting of distinct protein and lipoid particles, because in a polyphasic system the superficial layer is always formed of the continuous phase which, as we have seen, in cells is made of protoplasm.

Theoretically, there may be an inversion of phases in a polyphasic system, the internal or disperse phase becoming (and *vice versa*) continuous or external; and consequently, in living cells an external phase, for example a lipoid one, passing over to form the cellular membrane. But, in fact, in normal cells no fatty or lipoid phase exists phasically differentiated from a protein

[38] P. Scherrer, "Bestimmung der inneren Struktur un der Grosse von Kolloidteilchen mittels Rontgenstrahlen," in R Zsigmondy's "Kolloidchemie," pp. 387-409, Leipzig, 1920. *Idem., Nachr. Kgl. Ges. Wissensch. Gottingen*, 98, July 26, 1918.

[39] H. Handovsky writes (*loc. cit.*, p. 60): "By means of this method, Scherrer (in Zsigmondy, "Kolloidchemie," 3rd ed., Leipzig, 1920) showed that in particles of colloidal dimensions there are the following kinds of form: Colloidal silicic and stannic acids are amorphous substances with many crystalline inclusions; gelatin is a mixture of two amorphous substances; starch and cellulose are crystalline. By the same method R. O. Herzog and Jancke have shown that glycogen is amorphous, silk is crystalline, viscose (a cellulose ester) is amorphous."

Liquid crystals are " 'semi-isotropic' structures, in which the molecules are oriented parallel in one plane, while remaining at random in the other planes. By the use of external forces acting on amorphous substances (with crystals the interior is always concerned), we can always produce vectorial conditions; thus unquestionably amorphous substances like glue (Brewster, 1816), plate glass (Mach, 1873), stressed rubber (Ebner, 1882) may be made negatively or positively doubly refracting by pressure or strain, so that they transmit more or less light along the strain or pressure axis than in other directions. And it is probable that we have here a regular orientation of molecules, brought about by the pressure or strain, as Svedberg showed to be the case with iron hydroxide, when he examined it ultramicroscopically in a magnetic field."

[See also paper by G. Friedel, in Vol. I of this series. *J. A.*]

one, and therefore, the view held by few, that the "cellular membrane" can be at times formed by proteins and at times by fats and lipoids, is purely fantastic.

Concerning physiological solid or fluid homogeneous systems, we know that no important metabolic processes take place in them. No catalytic reactions of importance occur in blood plasma or in red blood cells. Heterogeneity would, therefore, not be advantageous; as a matter of fact, it would be detrimental, because the blood plasma is mainly a vehicle for nutritious substances and metabolic products, a solvent of these substances, and, as such, it is desirable that it should not contain granular-disperse phases, upon the surface of which these substances could be adsorbed. Similarly, the crystalline lens, hyaline cartilage, vibratory cilia, and the cuticle, the cellulosic walls of plants, etc., are structures that accomplish physical and mechanical tasks, and in which ordinarily few metabolic reactions take place. If they actually are solid homogeneous colloidal systems, we must agree that this is most convenient.

Admitting that the systems just mentioned are homogeneous, and that they therefore differ from metallic suspensoids, it appears proper to me that in homage to the memory of the one who first used the name to designate chiefly substances peculiar to animal and plant organisms, the term *colloids* (from κολλα, meaning precisely in the language of Aristotle, *glue*) be reserved to the natural colloidal systems of living organisms; and that we apply the name *suspensoids* to metallic and inorganic, as well as to artificial organic protein, lipoid, etc.) disperse systems. These could be divided in hydrophobe and hydrophile according to the affinity of their disperse particles for water.

Their precipitates or dispersible coagulums could be called *hydrogels* and *jellies*, the solid not easily dispersible coagula. As far as the size of the disperse particles is concerned, natural colloids lie between suspensoids and the molecular- and ion-disperse systems of simple inorganic substances (crystalloids of Graham), just as suspensoids lie between colloidal systems (according to definition given) and coarse suspensions. But as far as the physico-chemical properties are concerned, colloidal systems (in the sense just mentioned) are more closely related to solutions of crystalloids, just as the suspensoids are more closely related to the suspensions.

Amongst the natural colloids we differentiate: (1) liquids, e.g. the blood plasma; (2) those highly viscous, e.g. the protoplasm, the karyoplasm, the content of erythrocytes, etc.; (3) solids, e.g. the crystalline lens, fibrillar formations, etc. Now, to designate each of the groups by one word, I propose to call the first simply *liquid colloids*, the second *gliodes* (from γλοιώδης = glutinous, tenacious, viscous), and preeminently *gliode*, recalling "sarcode," the protoplasm, the continuous phase of the cytoplasm; thirdly *solid colloids*, the crystalline lens, hyaline cartilage, connective and elastic fibrils, etc.

The classification of the disperse systems could therefore be modified as shown in the accompanying table.

Disperse Systems.

I. *Suspensions.* (Particles in suspension, visible in the microscope, called *microns.* Systems liquid, turbid.)

(1) *Suspensions* proper (of solid particles).

(2) *Emulsions* (suspension of fluid particles).

II. *Suspensoids.* (Particles in suspension, visible with the ultramicroscope, called *submicrons;* particles of metals and metalloids, of organic and inorganic substances. Liquid systems, usually artificial.

(3) *Hydrophobe* or *lyophobe suspensoids* (containing particles having little or no affinity for the dispersive medium. *Suspensoids,* in strict sense).

(4) *Hydrophile* or *lyophile suspensoids* (containing particles having more affinity for the dispersive medium. *Emulsoids,* in strict sense).

III. *Hydrogels* and *jellies* (solid systems).

(5) *Hydrogels.* (Precipitates and coagulums of suspensoids and emulsoids mechanically dispersible, and, early in certain cases, reversible; formed by the aggregation of the disperse phase, with massive separation of the liquid phase.)

(6) *Jellies.* (Compact masses not easily dispersible by mechanical means, apparently homogeneous, in which the liquid phase is retained in the framework or the alveoli of the solid phase).

IV. *Colloids.* (Natural colloidal systems, whose disperse particles, at least partly made of molecules and ions of organic substances, are much hydrated and not distinctly visible in the ultramicroscope, but often capable of producing a diffuse luminosity. Systems, at least some of them, optically and physically homogeneous.)

(7) *Liquid Colloids.* (Solutions, at least partly molecular and ionic, of substances colloidal *par excellence.* Moderately viscous liquids, as the blood plasma, lymph, etc.).

(8) *Gliodes.* (Colloidal systems, fluid as the preceding ones, but extremely viscous, as protoplasm.)

(9) *Solid Colloids.* (Condensed colloidal systems, more or less transparent, usually birefrangent, that probably show a meta-structure on X-ray examination; e.g. the crystalline lens, connective and elastic fibrils, cellulose cell walls, vibrating cilia, etc.)

V. *Molecular* and *ionically disperse solutions* (of crystalloids, whose particles are called *amicrons,* because, being extremely small and much hydrated, they are invisible in the ultramicroscope. Homogeneous liquid systems optically void).

Proteins as Colloids

By Prof. Dr. Wolfgang Pauli,

University of Vienna *

The most characteristic constituents of an organism form an enormous group whose general properties show a marked resemblance to inorganic colloids. In fact, to-day colloids may be regarded as an important, perhaps the most important connecting link between the organic and the inorganic world. At first this connection leads to the purely chemical and physical aspect of life processes, but since, as we shall see directly, it throws new light on basic questions such as the innermost structure and organization of chemical trans‑ formations, it opens up new approaches to the study of the living cell.

The most striking characteristic of a stable inorganic colloid, e.g., ferric hydroxide sol, is the *polymolecular* constitution of its particles; for the colloid particles are from 3 to 300 times the diameter of a simple molecule, which means that there are up to about 36 million molecules per particle. A second characteristic is the *maintenance of a definite degree of dispersion;* that is, in a stable sol, in the course of time there is no re-solution of particles into simple molecules, nor is there progressive separation by growth or aggregation of the particles, as in precipitate formation. Colloids are thus intermediate between soluble and insoluble substances, or to put it another way, they combine the properties of both to a certain extent. What is the basis of such behavior?

It accords more with our present state of knowledge to consider what colloids have in common with other substances, rather than the points or peculiarities wherein they differ. We therefore give a general survey of the phenomena and the revolutionary modern views on atomic structure, which have placed chemistry and physics on a new basis.

As Rutherford and Bohr have shown, atoms consist of a tiny positive nucleus, about which revolve in planetary fashion, negative electrons, elementary units of (negative) electricity. The volume of the atom is not determined by the mass of the electrons, each of which is one two-thousandth that of the lightest element, hydrogen, but by the much greater amplitude of the paths of the electrons. The number of electrons in an element increases with increasing atomic weight, and corresponds with its atomic number N in the periodic system of the elements. Thus the sixth element, carbon, has six planetary electrons, and the last, uranium, has 92 of them.

Certain numbers and arrangements of the electron paths form particularly stable groupings. Such elements constitute the noble gases, helium (Se), argon (A), neon (Ne), etc., whose electronic stability finds expression in great chemical inertness, in their inability to form compounds. Elements intermediate between the noble gases are much more reactive. Walter Kossel believes this is because they tend to higher or lower stable electron configura-

* Translated by Jerome Alexander.

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tions of the higher or lower noble gases. That is, as soon as conditions permit, they take or give up a sufficient number of electrons to complete an electron arrangement like that of an adjoining noble gas. For example nitrogen has the atomic number 7, and the next higher noble gas neon has the atomic number 10; therefore in order to reach the stable noble gas configuration, nitrogen must take up three electrons, or three negative charges, as instanced by its valence in ammonia, NH_3 (Fig. 1). The noble gas immediately below nitrogen is helium, whose atomic number is 2, so that to resemble helium, nitrogen must be able to give up five electrons, which means that it must yield an ion with five positive charges, as is the case in the formation with oxygen of nitric acid, HNO_3, which carries two negative charges (Fig. 1). In numerous instances we can see thus that ion formation from atoms occurs in such fashion that two elements exchange electrons, the giving up of electrons, to form positive ions on the one hand balancing the taking up of electrons with formation of negative ions on the other. Compounds of

ORIENTATION OF A DIPOLE ABOUT AN $N^{\pm}$ ION
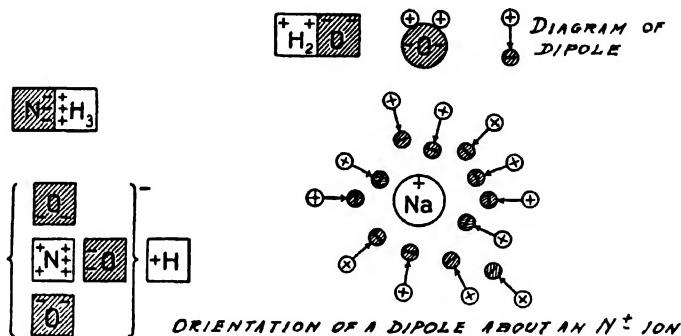
FIG. 1.   FIG. 2.

this kind may be conceived of as being held together by the electrostatic attractions of their ions. They are called *heteropolar* compounds.

In what follows we will initially treat ions as charged spheres surrounded by an electric field.* Consider such atomic monovalent ions, e.g., Na^+ and Cl^- formed by solution of sodium chloride in water. The water molecule is heteropolar, since the O-ion with two negative charges is combined with two positive H-ions to form a molecule electrically neutral externally. It will, nevertheless, be acted on in an electric field. The positive and negative charges of a heteropolar molecule may be conceived of as concentrated at a mean point, as with the center of gravity in masses of matter. If the individual charges are exactly of the same shape, or as we may put it, spatially isotropic, with respect to each other, then the positive and negative mean points coincide. Where this is not the case, we have *dipoles*.

Numerous experiments show that the water molecule is such a dipole (Debye), as is shown in Figure 2. These dipoles will be oriented in the fields of the Na- or Cl-ions of sodium chloride, presenting their negative ends to the positive ions and their positive ends to the negative ions (Fig. 2). Any movement of the ions must therefore cause a twisting of the dipoles. This

* Most molecules are far from spherical. See "The Effects of Molecular Dissymmetry on Properties of Matter," by Irving Langmuir, Vol I, p. 525. *J. A*

twisting of the dipoles is, however, opposed by the internal friction of the water, whereby there must result a consequent electrostatic braking action on all ions. A group of experimental facts, which have heretofore been regarded as indicating ion hydration consequent on the formation of actual compounds of ions with water, are explained, and to some extent quantitatively, by this electrostatic theory advanced by M. Born. We further know that under like conditions increasing charge or valency of ions involves stronger hydration of this kind, and also that, *caeteris paribus*, ions of smaller volume exert at their surface a stronger electric field, that is, a stronger polarization of the water dipole, than do ions of larger radius.

If two oppositely charged ions approach in solution, the water dipoles lying between them will try to orient themselves in opposite directions. Under suitable conditions these tendencies balance each oher. There results a more or less complete mutual *dehydration*, the ions being deprived of water. If we increase the concentration of the electrolyte, e.g., continually increase the content of Na- and Cl-ions, more and more and oppositely charged ions come into the range of molecular activity and dehydrate each other. From
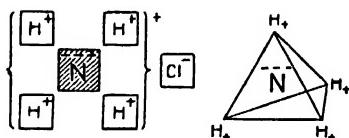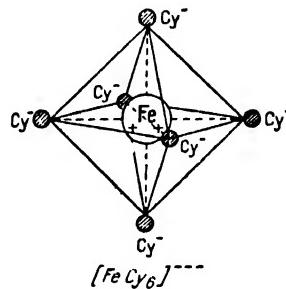
Fig. 3.   Fig. 4

a supersaturated solution, the ions finally separate out as crystals. That crystals of heteropolar compounds are built up of ions which are set definite distances apart and at fixed points in the so-called space lattice, is a most important fact revealed by Roentgen ray analysis (X-rays). We therefore see that the union of ions and their separation from solution must be regarded as a dehydration consequent on the action of Born's dipole.

This type of formation of a crystalline precipitate is, therefore, one way in which, at least from a chemical point of view, a polymolecular structure is built up. But heteropolar molecules may unite another way. Let us consider a simple case, the reaction between the two heteropolar molecules ammonia and hydrochloric acid, $NH_3 + HCl = NH_4Cl$. The NH_4Cl formed breaks up in solution into the ions NH_4^+ and Cl^-. According to W. Kossel's views, this may be expressed as follows: Groups of positive H-ions have accumulated in the powerful electrostatic field of the trivalent N with this latter as central ion, while at the same time the negative Cl-ion is repelled from the similarly charged N^{---} and completely dissociated (Fig. 3). Following Werner, we call such molecular combinations *complex compounds*. In contradistinction to simple or elementary ions, NH_4 is a *complex* ion.

The formation of a complex ion demands the presence of sufficiently powerful field, in which a stable grouping of oppositely charged ions (or their

mean location) is possible. The central ion occupies the center; four co-ordinated surrounding ions may be conceived of as occupying the corners of a tetrahedron; six, the corners of an octahedron; eight, the corners of a cube. And, in fact, these are the most usual coördination numbers of ions in the first sphere surrounding the central ion.

A second example will give us another aspect of complex formation. Bi-valent ferro-ions form with cyanogen ions the insoluble ferrous cyanide, $Fe(CN)_2$. This dissolves in an excess of KCN to form an alkaline ferro-cyanide according to the equation (Fig. 4)

$$Fe(CN)_2 + 4KCN \rightarrow [Fe(CN)_6]^{----} + 4K^+$$
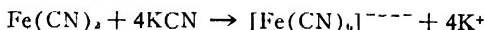
Thus is formed the complex ferrocyanide ion with four negative charges. In this case an insoluble molecule, $Fe(CN)_2$, has been transformed into an ion; it has acquired a powerful charge from the cyanogen ions entering the field of the ferrous ions, and in consequence of the incidental powerful hydra-tion, there has resulted a very soluble compound. To a certain extent, this picture is typical for the formation of most inorganic colloids.

We shall continue our consideration of the structure of colloids, for we desire to examine the principal cases, insofar as may be necessary for a review of colloid reactions. Ferric hydroxide is a case that has been much studied. If we mix ferric chloride and caustic soda, Cl is replaced by OH with the formation of insoluble ferric hydroxide, according to the equation

$$FeCl_3 + 3NaOH \rightarrow Fe(OH)_3 + 3NaCl.$$
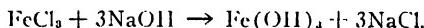
A similar change occurs even upon simple dilution of the salt—

$$FeCl_3 + 3H_2O \rightarrow FeOH_3 + 3HCl.*$$
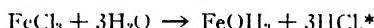
This so-called hydrolysis can be increased by removing the hydrochloric acid formed, as by dialysis in a parchment paper sac, but the process is stopped before all the Fe is converted into the insoluble hydroxide. The sac then contains a clear brownish red sol. If sufficiently purified, it is free from H-ions and generally contains from 20 to 50 times as many Fe atoms as Cl atoms. All the Cl is nevertheless available for chemical reactions, and is therefore at the surface of the particles, but only about 20 to 25 per cent of the total Cl appears to be active in conductivity or other (e.g., electro-metric) measurements.

We may conceive these colloid particles to be built up as shown in the diagrammatic Figure 5, with a nucleus of $Fe(OH)_3$ carrying at its surface ionizing molecules of FeOCl. There are present, therefore, two constituents; one, the essentially insoluble $Fe(OH)_3$, the other, the ionizing solubilizer, FeOCl.† The particles show a positive charge, and flocculate on being discharged, e.g., at the negative

⊞ ●
FeO.   Cl = *Iogenic Complex*
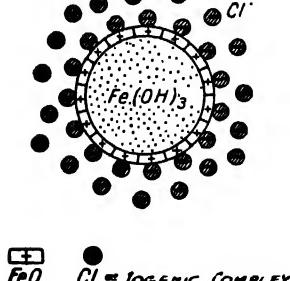
Fig. 5.

pole of an electric circuit or on radiation with β-rays of a radioactive substance. Corresponding to the positive charges on the surface of the colloid particles are a like number of circumambient negative charges, which we will call *contraions,*—in this instance, Cl-ions. The charge also controls the behavior toward the solvent, leading to a stable degree of subdivision. This type of structure, an insoluble portion charged and stabilized by numerous ionizing superficial complexes, is quite generally evident with inorganic colloids, and is also found in sols of the noble metals and in organic colloids, among them a numerous group of proteins.

Colloid ions, as we learn from physico-chemical analysis, usually carry many charges on their surface—it may even be thousand or hundreds of thousands—and the electric field produced is greater the greater the charge is on the same size particle.* A high electric charge leads to increased electrostatic reaction with the water molecules and to an increased hydration, which, under favorable conditions is observable in an increased viscosity of the sol.† Thus an increase in the charge of the particles may give rise to an increase in viscosity, and a decrease in charge may bring about a drop in viscosity.

If, however, the colloid ions have a large number of charges, we may then expect their electrical reaction with other ions to give rise to many phenomena. But out of this fact there arise many explanations.

According to recent knowledge and views, all ions in the solution of a salt are equally free, but in conductivity or free ion measurements their total number is not active, because part are braked or inactivated by the attractive forces of the oppositely charged ions. (Bjerrum.) If we continue our diagrammatic representation of a positive colloid ion, we find drawn about and held to such ions because of their fields, a certain number of oppositely charged ions of which, however, a fraction takes no active part—e.g., with ferric oxide sol this fraction is 20 per cent of those ions concerned in conductivity, and 80 per cent of the Cl-ions corresponding to colloid particles. Increasing the Cl-ions in the fluid, e.g., by adding sodium chloride, increases the Cl-ions in the field of colloid particle and leads to its electrostatic inactivation. Finally, because of this supersaturation at the surface of the colloid particle, they combine firmly with it in ever-increasing numbers, are adsorbed by it. The colloid particles gradually lose their charge, until they fall out of solution. This is the mechanism of colloid precipitation by electrolytes as we must now picture it according to the theory of interionic forces in electrolytes. (Bjerrum.)‡

We may now take up the case of the *proteins*. In complete absence of electrolytes, effected by careful electrodialysis, proteins show a faint conductivity and a very slight migration in an electric field; they therefore possess a very small definite electric charge. They nevertheless react, in aqueous solution, as amino acids of the type $NH_2 . R . COOH$, as well as like acetic acids with their $COOH$ groups, and also as bases like ammonia because of their NH_2 groups. Hence we have the equations

$$NH_2 . R . COOH + NaOH \rightarrow Na^+ + NH_2 . R . COO^-$$
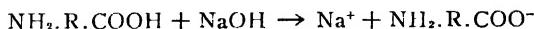
* R. A. Millikan's colloidal oil droplets carried over one hundred negative charges (electrons). See Vol. I, p. 181. *I. A.*
† For the approximate limits within which this is so, see Vol. I, p. 19. *J. A.*
‡ We must also consider the very important consequence of loss of charge—aggregation into groups of such size that their kinetic motion is insufficient to keep them afloat. For a more detailed consideration of coagulation, see II. J Kruyt, Vol 1, p. 306. *I. A.*

for the formation of negative protein ions, and

$$COOH.R.NH_2 + HCl \rightarrow COOH.R.NH_3^+ + Cl^-$$
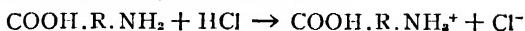
for the formation of positive protein ions.* R stands for the rest of the protein molecule. A further important result is that with increasing addition of acid or alkali not merely one, but many NH_2 groups are available for reaction, and likewise a larger number of carboxyl groups to combine with alkali. The charge of the individual protein ions therefore rises upon addition of acid or alkali, even to 40. Since, however, the proteins are composed of molecular chains containing many atoms, they have a colloid character, and must be considered, when highly charged as acid or as alkali protein, to be similar, with reference to the field activity of their ions, to the electropositive or electronegative inorganic colloids. Thus when we add HCl to a protein, at first practically the whole amount of acid added reacts with the formation of an ammonia salt. Therefore ion formation and e.g., the conductivity portion of the salt, as well as its activity, will grow until the protein
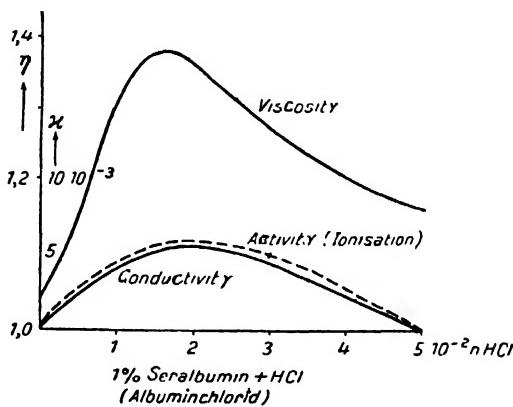
Fig. 6.

ions have almost reached their full charge. If we add still more HCl, it can no longer increase protein ionization, but merely increases the Cl-ion concentration in the field of the highly positively charged protein ions. As a consequence of the increased electrostatic reaction, increasing numbers of Cl-ions are inactivated and finally firmly bound by the protein, a process which is, as we know, followed by dehydration. The dehydrated protein is finally flocculated, just like a positive ferric hydroxide sol by an excess of a chloride. *This is the way proteins are coagulated by strong acids.*

The process may be followed quantitatively. If the H- and Cl-ions in an acid protein mixture are determined by an electrolytic chain, we get an activity curve of the protein, which gives a picture of its state of ionization. With increasing addition of acid the curve must first rise because of increase in protein ionization, and drop again with excess of HCl, because of inactivation of the Cl. In like manner, the conducting portion, the conductivity of the protein salt, must first rise and then fall. This is shown in Figure 6

* Since deaminized gelatin still binds acid, it is extremely questionable if this convenient explanation represents the actual facts, though it may represent a tendency sometimes reached. See Vol. 1, p. 547, et seq., also "Glue and Gelatin," by J. Alexander, Chemical Catalog Co., **1924**, p. 32, et seq. See especially, paper by T. Brailsford Robertson, this volume. *J. A.*

giving the activity and conductivity curves of serum albumin, based on determination made with Frisch and Valkó. The initial increase and subsequent drop in protein charge shows itself in the hydration, and in our case also in the viscosity. As the figure shows, the maximum viscosity coincides with the maximum in activity and conductivity.* The end of the activity curve corresponds to a gradual acid flocculation of the albumin. Other proteins exhibit the same behavior. When we furthermore consider that ionized, strongly hydrated protein, is less sensitive to alcohol precipitation and heat coagulation, while the reverse is the case with the de-ionized protein, it is obvious that the whole gamut of protein properties is reflected in its electrostatic behavior.

The characteristic maxima in the properties of acid proteins, just mentioned, indicate solutions in which the protein ions are free to move in all directions in the solvent. Here there is no question of any opposition to their diffusion. As is well known, the Donnan equilibrium, now so often quoted to biologists and physicians, depends on the fact that colloid ions, enclosed or otherwise fixed in a cell impermeable to them, show a different distribution from the other accompanying movable ions, which in simple cases may be easily calculated thermodynamically. J. Loeb therefore considered the whole colloidal behavior of proteins as a secondary consequence of this distribution of ions, and therefore as referable to the principle of Donnan. But in all the cases we have been considering, this is not so, for there is no question of any interference with the diffusion of colloid ions in the protein solution. According to the clear and simple exposition of Donnan, this interference is an essential preliminary to the application of his principle. Loeb's approximately harmonious calculations and incidental corrections are furthermore erroneous, because they leave out of consideration the very important and varying activity. The conductivity as well as the osmotic activity of the protein is dependent on the electric attraction of its contra-ions, and therefore its osmotic pressure will parallel its activity whose course, as we saw, also indicates the viscosity.

Figure 7 gives such curves of osmotic pressure and viscosity of albumin with increasing acid content, taken from a paper published in 1913; the curves clearly show this agreement. The fact that freely moving ions must distribute themselves between an osmotic cell and the exterior fluid according to the Donnan equilibrium when the conditions for such distribution are present, is thermodynamically a necessity, which does not primarily determine the properties of proteins, but which, quite to the contrary, is intimately dependent upon changes in proteins. Loeb's explanation of the colloidal properties of proteins, which we believe to be basically erroneous, has been widely extended. How far the application of the Donnan equilibrium to the organism has been driven, based on Loeb's views, is evident not only in the numerous applications to parthenogenesis, but also in the attempt to explain thereby even therapeutic activity, such as that of Roentgen rays (X-rays).

In the case dealt with above, the reaction of the colloid or highly charged protein ion with *monovalent* ions was considered. If *polyvalent* oppositely charged ions are allowed to react with the colloid, the mutual electrostatic reaction is considerably intensified. If to our positive ferric hydroxide sol, whose contra-ions are monovalent Cl-ions, we add divalent negative sulfate

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* These experimental results are excellent evidence in favor of a somewhat different aspect, involving a zone of maximum colloidality, consequent upon variations in degree of dispersion. The two views are not inconsistent with each other. See: Vol. 1, p. 19, *et seq. J. A.*

ions, the latter will be more powerfully attracted by the colloid ions and the similarly but weakly charged Cl-ions will, by repulsion, be promptly driven out of the field. Stronger electrostatic attraction indicates stronger association of the sulfate ion with the colloid, which flocculates when all the Cl-ions are discharged by SO_4-ions.

With acid protein, a higher charge of the contra-ion finds similar expression. Sulfate ions reduce the conductivity more than do Cl-ions, and the stronger de-ionization of the protein ions is reflected in the diminished hydration and viscosity of protein sulfate. Protein sulfate is actually more readily flocculated than the chloride by excess of acid, as well as by addition of alcohol. Figure 8 gives the viscosity and conductivity of gelatin (glutin) chloride and gelatin sulfate with increasing acid content according to the experiments of H. Hugowit, and the difference is quite evident. Still more highly charged negative ions must be still more powerfully held electrostatically by acid albumin, and being about dehydration and flocculation. Such is, e.g., the quatravalent ferrocyanogen ion $(Fe[CN]_6^{----})$. On this
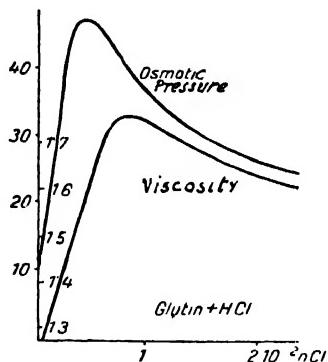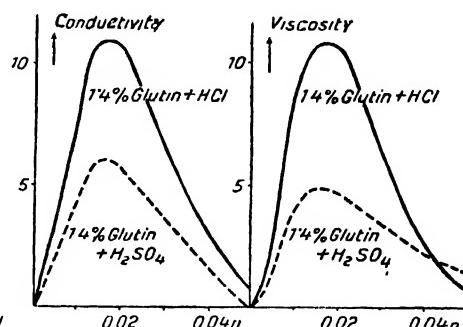
FIG. 7.   FIG. 8

depends the extreme sensitiveness of the ferrocyanide-acetic acid test. The acetic acid insures a positive charge, the ferrocyanogen ion the discharge and flocculation of the albumin.

The reactions of alkali proteins present quite the contrary picture to this flocculation. As per the outline given above, the protein is converted into a highly charged negative colloid ion. As we have shown years ago, within corresponding limits, in this case too an excess of alkali will precipitate the protein, just as with acid flocculation. But results are better if contra-ions of higher valency are used. Especially active are ions of the heavy metals, like Ag^+, Hg^{++}, Cu^{++}, Fe^{+++}, etc., which, partly because of their small atomic volume, partly because of their higher valency, give rise to increased electrostatic reaction. These properties form the basis of important practical methods for removing proteins.

The increased activity of field in colloid ions in addition to the effects due to the valency, the volume, and the hydration of the contra-ions present, suffice to explain in general the colloid reactions heretofore referred to. There is, however, a large group of ions which are exceptional, in that volume and charge would lead us to expect a much lower colloid precipitation capacity

than is actually found. Thus according to experiments of H. Freundlich, to whom we owe the first experiments of this kind, the coagulating power (measured on negative arsenic trisulfide) of the monovalent positive ions of guanidine nitrate, strychnine nitrate, morphine chloride and New Fuchsine is from 3 to 50 times as great as that of the monovalent potassium, or other alkali metals, and always from 2 to 30 times as great as that of the H-ions of strong acids, despite the small volume of the latter. Freundlich further showed that the ions in question were also readily adsorbed by certain fine powders.

At first glance we see that we may have here what is perhaps no accidental group of physiologically interesting substances. In order to review the active forces possibly involved, we will consider what seems to be a somewhat isolated simple case. It has long been known that the small-volumed H^+ and OH^- ions, that is acids and bases, are more active in causing the flocculation of negative or of positive ions than K^+ or Cl^- ions, which likewise are monovalent. We chanced upon an experiment which showed that here

OH - *Ions in the field of a positive colloid ion*
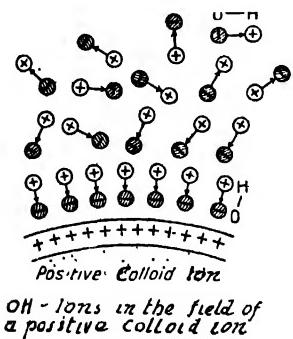
FIG. 9.
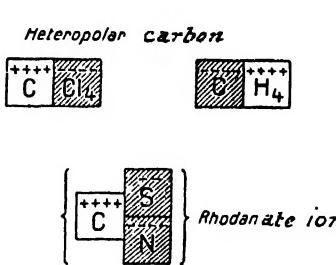
Fig 10.

small volume alone could not be the critical factor. We showed that the most diverse and negative colloids heretofore tested, including even sols of the noble metals, could be converted into colloids wherein H-ions served as contra-ions for the colloid particles. A certain amount of free H-ions is therefore capable of existence in the presence of negative colloid particles. On the other hand, it was under no circumstances possible to prepare a positive sol having simple negative OH-ions in the solution as contra-ions. In this case the sol precipitated, like our ferric hydroxide sol when its Cl-ions were replaced by divalent sulfate ions.

In order to understand this behavior, it would seem, at present, that the charge of an OH-ion is distributed very eccentrically, that the divalent negative charge of O^- and the single positive charge of the small H-ions form a pronounced dipole. At larger distance the ion with its excess negative charge may well be considered as a point charge. But within the range of molecular attraction of a positive ion, the OH may be turned or oriented as shown in Figure 9, the divalent negative O^{--} turning toward the positive colloid ions.* The electrostatic attraction is thereby considerably increased, and

* See e.g. Irving Langmuir's paper   Vol I entitle "The Effect of Molecular Dissymmetry on Some Properties of Matter." *J. A.*

some of the divalent negative ions may approach very close to the colloid ion.

Therefore in cases where the ionic charge is *eccentrically* distributed, we must allow for the fact that the ion will be turned or oriented in the electric field, with a consequent change in electrostatic relationship on entering the range of molecular attraction. This orientation may be termed *electroversion*. Using a different case, the formation of surface films, on water by capillary active substances, e.g., by fatty acids, the American investigators Langmuir and Harkins * assumed and experimentally demonstrated the orientation of large molecules.

We will now consider several remarkable cases rendered comprehensible by the *electroversion of ions*. Because of its central location in the periodic system, carbon readily gives up four exterior electrons or else takes up four additional electrons. When strongly heteropolar, carbon is either positively quatravalent, e.g., in CCl_4, or negatively quatravalent as in methane, CH_4.
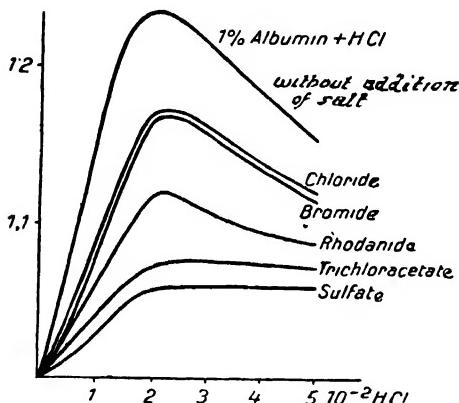
Fig. 11.
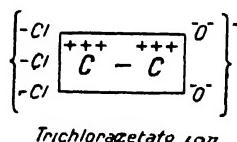
Fig. 12.

Consider now the negatively monovalent sulfocyanide ion SCN, which occupies an anomalous position with respect to proteins as well as physiologically (Fig. 10). Here C is in a strong heteropolar combination as a positively quatravalent ion combined with negatively trivalent N and negatively divalent S, leaving a single free negative charge. The figure, however, shows the markedly eccentric distribution of the charge in this complex ion. In a strong positive field it must, therefore, be so oriented that the negative N and S-ions are turned toward the positive charge, and positive C away from it. The eccentricity of the ionic charge is thus increased in the field. Accordingly, under favorable conditions we must expect an increased reaction between sulfocyanide ions and positive colloid ions, over and above what is indicated by its single negative charge. Figure 11 shows first the viscosity of 1 per cent serum albumin with increasing acidity, which, as we know, first reflects the positive charge of the protein salt formed. If we increase the negative ions in the field of the positive albumin, e.g., by addition of an equal concentration of the corresponding salt, the charge and also the viscosity of the protein ions diminish. The powerful depressing action of sulfocyanide is

* See papers   ch of these investigators in Vol. I. I A.

obvious, surpassing that of chloride or bromide. It is surpassed by the monovalent trichloracetyl ion, which is nearly as powerful in inactivation and dehydration as the divalent sulfate ion. Trichloracetic acid is well known to be a splendid and much used protein precipitant, and as is shown by Figure 12 exhibits a very eccentric negative charge, the oxygen ions turning toward the positive colloid ion. It is obvious that a strongly positive central field dominates the trichloracetyl ion, and that the strong repulsion is favorable to the dissociation of the H-ions. Indeed trichloracetic acid is one of the strongest organic acids, so that it charges proteins even in slight concentration, and precipitates them even in slight excess.

To save time, we may refer to the accompanying figures, which show first the eccentric distribution of charge in the guanidine ion which is markedly heteropolar, and second in pyridine, whose N, despite its location in the pyridine ring, keeps its residual negative charge in active state, so that pyridine is an excellent precipitant for acid proteins. (H. Handovsky.)

For comparison we include tetraethylammonium, another organic ion having an isotropic charge distributed in a tetrahedron. This ion fails to show any of the peculiarities exhibited with proteins by eccentrically built ions.
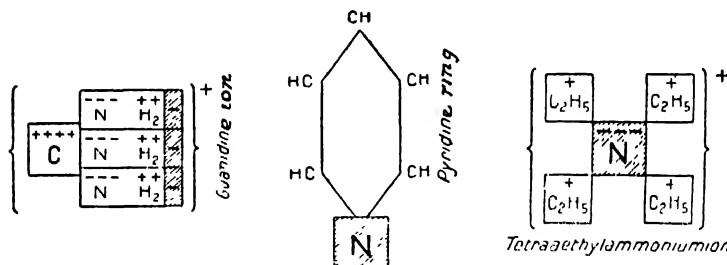
Fig. 13.

Strongly charged positive protein ions are, furthermore, such good tests for eccentric distribution of charge in negative ions, that with their aid under suitable technique, such eccentric structure may be demonstrated in the more simply constituted acetate, NO_3-, and ClO_3-ions. In alkaloids and dyes of high molecular weight, the free valence is placed eccentrically, and this favors orientation of molecules involving increase in charge and a confluence of the major portion of the molecule. It is evident then that negative dye ions are most sensitive reagents for positive proteins, while positive ions of dye bases are sensitive reagents for negative proteins.

We must here leave this discussion, which has been mainly directed to highly charged protein ions. The somewhat different but not less important properties of proteins when near electro-neutrality—in the so-called isoelectric zone—must be treated elsewhere. We may say, however, that the same principles serve to explain their behavior, if we give due weight to the weak charge and to the amphoteric character of proteins.

To sum up, many colloid and protein reactions are due to electrostatic interaction, providing that proper consideration is taken of *the relation between chemical configuration* and *the spatial distribution and intensity of the electric fields.* From this there follows, always on the basis of the same electric forces, an extreme diversity and differentiability of reactions, which, as may

readily be shown, may in each case be brought to the degree of *specificity*. The specificity of chemical relation is, however, held up before biochemists and pharmacologists as a fundamental problem. It is manifested exclusively through colloid reactions, either by added crystalloids acting on the colloids of the organism, or by colloids acting on each other.

We can now understand why the organism generally blocks any tendency of its colloids, especially its proteins, to acquire a stronger charge. For the resulting strong electric fields, apart from increased hydration, must militate against specificity, in that they would finally reduce all colloids of the same kind of charge to the same level. Furthermore strongly charged protein in the organism would, as a defensive measure, accumulate a relatively high concentration of ever-present salt ions, with the result that there would be effected, especially by polyvalent ions, an inactivation and neutralization of the protein charge. This is, in fact, an important general compensating function of salts, which * may, in individual cases have special significance. The intermediate reaction of the tissue juices maintained by a delicate regulation of the H-ion concentration, may be considered as preventing strong charge of proteins, and preserving sensitivity to specific reaction.

A tissue acquires specific affinity generally because of having cells in rigid structures, and rigid structures form, in this case, oriented molecules which are eccentric and have their feeble charges directed toward the medium, but which may otherwise be tied together. On the other hand, for the formation of chemically inert structures having little to do with interchange of material as is the case with various supporting substances, the electric fields must be neutralized as far as may be, as is possible, e.g., by mutual co-deposition of amphoteric molecules with their positive, negative, dipolar charges. Such molecules predominate in proteins near their neutral point. Residual charges may, outside of this, be satisfied by polyvalent ions, as may be assumed, e.g., in osseous structures by Ca^{++}, phosphate, and carbonate ions, to a certain extent.

This brief discussion clearly points out the direction that must be followed by biochemistry of the future, which must more and more be based on harmony with modern physics. In the not far distant future, such biochemistry will include a true electrical submicroscopic structure of living cells, which is something quite different from certain strained explanations of an antiquated, gross, electro-physiology.

* See e.g. Martin H. Fischer, "Edema," New York, 1909. Also his paper in this volume. *J A.*

Lyophilic Colloids and Protoplasmic Behavior

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I. Introduction

When it was established in the middle of the last century that all living things, animals as well as plants, are composed of cells, it became the primary purpose of all physiological workers to analyze these. This analysis may be said to have taken three directions—a purely chemical one which contented itself with the discovery that all living matter is composed of proteins, carbohydrates, fats, salts and water; a physico-chemical one which tried to rediscover in mixtures of this type the dilute solution laws of the physical chemists; and a colloid-chemical one which, with growing intensity, has emphasized the importance of the peculiar state of the chemical materials found in living matter for its behavior under normal and abnormal circumstances.

Any debate regarding the chemical items found in cells may be said to be at an end. The question at issue is their relationship to each other and here most biological workers still believe that the dilute solution laws of the physical chemists, properly utilized, will give us answer. A minority hold that this is not the case but that the laws, still but imperfectly known, which govern colloid systems will be the really important ones in unravelling the physiological puzzle.

Expressed in simple terms, what sort of system does the majority hold the living cell to be? For them, it is essentially a drop of water. In this they assume to be dissolved the various salts found in protoplasm, as well as various non-electrolytes like sugar and urea. A third place is assigned protein, which the pure chemists always mention first because they find it the characteristic element of all living matter. The fats and the carbohydrates—especially the higher ones—come struggling after, as additional materials which, with the proteins, are "suspended" in the water. To this droplet of mixed materials the physical chemists add a further element which they do not define in chemical but only in physical terms. This is the "membrane" that surrounds the droplet and which, depending upon the author, is "semipermeable," "plasmatic" or "fat-like."

In equally simple terms a colloid chemist would draw his picture quite differently. He would first wipe out the "membrane" because entirely hypothetical. The proteins he would place first. Second he would mention the salts, but not as things which are merely mixed with the protein, but as materials which, as acids and bases, were originally united with the protein. Third he would put the water, not as a solvent *for* the protein-salt complex, but as a material dissolved *in* the latter. This membraneless hydrated protein-salt compound is the unit of his living mass. Into it he mixes (emulsifies) the fats and the higher carbohydrates found in the cells.

Having in this dogmatic fashion set the points at issue opposite each other we may look at the evidence to see which side has the advantage.

II. THE OSMOTIC THEORY OF WATER ABSORPTION BY PROTOPLASM

I know of no better way of outlining the arguments at stake than to go back to the years preceding 1907 and sketch the view of water absorption by protoplasm to which we all subscribed then—and the authoritative workers in this country still subscribe. Aside from some "physiological" theories —which explain nothing—and the "pressure" theories born of the pathologists, all the biological workers of that time were dominated by the osmotic notion of water absorption. This osmotic notion, as first advanced by Pfeffer and de Vries for vegetable cells and developed shortly thereafter by Otto Nasse for animal cells, was a clean-cut physico-chemical concept. Since later workers have added nothing which is not merely a modification of this concept or, worse, a something which has confused the whole picture, it is well to get clearly in mind just what these first laborers in the field thought.

Concretely expressed, they held every cell to be a circumscribed mass of protoplasm surrounded by a semipermeable wall. The semipermeable wall was *by definition* impermeable to all dissolved substances but permeable to the solvent, water. Protoplasm they conceived of as essentially water in which there were "dissolved" various salts and non-electrolytes like sugar and urea. To materials like protein, lipoid and starch or glycogen they gave little attention, for these were incapable of exerting "osmotic pressure." According to the beliefs of Pfeffer and de Vries, these cellular units took up water whenever immersed in distilled water or any solution possessed of an osmotic pressure lower than the cell contents; or gave off water whenever immersed in solutions of higher osmotic pressure, according to the laws of osmotic pressure as enunciated later by Van't Hoff (on the basis of the numerical data of Pfeffer and de Vries' osmotic measurements) and modified by Arrhenius' concept of the electrolytic dissociation of acids, alkalies and salts.

It is well to consider why this clearly thought-out concept of water absorption as applied to protoplasm met with difficulties, for to overcome such may well be said to have been the sole object of all the workers who, since Pfeffer and De Vries, have tried to save the osmotic idea for physiology or pathology.

Difficulties arise from two sides, (1) from the purely biological and (2) from the purely physico-chemical.

From the biological side it is sufficient to point out that an osmotically constructed living cell is an impossibility. If surrounded by a semipermeable wall no dissolved substances can enter or leave it, which is to say that a cell could never take up its needed food, be it oxygen, salt, sugar or amino-acid; or rid itself of the products of its metabolism like carbonic acid, urea or phosphate. To meet such objection it is popular to say that the word "semipermeable" is not really to be taken so strictly. According to such compromisers the "wall" is permeable to many things "necessary" for the life of the cell; or it is permeable at one time and not at another, as the author wishes; or he makes it permeable in one direction but not in the opposite, etc. The thing yields a picture as complicated as the number of men who have busied themselves with the problem. In biological reasoning this fact remains: in proportion as the semipermeable membrane is made permeable to dissolved substances, concentration differences between cell content and

surroundings are equalized, and as this happens nothing remains to move water. *A living cell is, however, capable of taking up and giving off water and of taking up and giving off dissolved substances and these two things may occur at different times or at one and the same time, and with the solvent and the dissolved substances moving in the same direction or in opposite directions.* Whatever we may hold for or against any theory of absorption and secretion by protoplasm this remains certain: until it can explain *all* these biological traits it is not adequate.

From a physico-chemical standpoint the osmotic notion of water absorption by protoplasm fares even worse, for here the so highly prized "quantitative" experiments come into play—and fail. Were living cells true osmotic systems they should be equally affected, for example, so far as their volume is concerned, by iso-osmotic solutions of different salts. This they never are. If a sodium chloride solution of a certain strength just preserves the normal volume of a plant or animal cell, an osmotically equivalent solution of potassium chloride usually permits it to increase while the chlorides of magnesium, calcium or iron lead to shrinkage. Again, by the laws of osmotic pressure, unit increments in the concentration of any salt solution should lead to unit decreases in the volume of a cell; as a matter of fact the amount of shrinkage is progressively less with every such increment increase. Finally, certain substances, like the acids and alkalis, act upon living cells in contravention to all the laws of osmotic pressure. They make cells take up water until they have swelled not only beyond the bounds of their normally calculated osmotic pressure, but beyond such as has been calculated for them (Overton) on the assumption that all their proteins, carbohydrates and fats are split into smaller molecules through the acid or alkali.

The original masters in physiology who dedicated their lives' efforts to the establishment of the osmotic concept of water absorption by living cells were familiar with many of these facts, and therefore never held to the adequacy of their concept with half the passion of the more modern workers upon their idea. Pfeffer, for example, knew that the osmotic notion did not explain all his biological observations and suggested that "imbibition" might play a rôle; and Otto Nasse, intent on discovering whether muscle behaved as an osmotic system decided it did not, when he observed how intensely acids made this material swell. Franz Hofmeister in the early nineties held the "water attracting" action of salts upon gelatin plates to be comparable with the cathartic action of these salts in pharmacology, but his ideas were spurned by a generation which saw physiological progress only in the application to protoplasm of the physical chemists' dilute solution laws (which in major portion promptly proved themselves not to fit).

III. THE COLLOID-CHEMICAL THEORY OF WATER ABSORPTION

It was with the problem in this state that, in 1905, I turned to an experimental study of the quantitative value of the swelling of hydrophilic colloids, more particularly of the proteins, for the elucidation of the problem of water absorption by living cells under both physiological and pathological circumstances (edema). Two years of work yielded little to convert my hypothesis of colloid water absorption into an experimentally founded theory until, one afternoon, while busy upon a related problem, I discovered a fact which at once explained all the experimental findings which until then had stood

against the establishment · of a colloid-chemical theory of absorption and secretion. As the importance of the fact and its necessary corollaries are not yet clear to many of my critics, I inject this bit of personal history. I was trying to prove that peptic digestion under the influence of different acids was not, as then generally believed, dependent upon the action of the different acids upon the *ferment, but upon the protein,* and was looking for a parallelism between the order in which the acids affect proteolysis and the order in which they induce some visible change in the protein. With fibrin used as the protein to be digested, a parallelism between rate of digestion and degree of its swelling was quickly established. In trying, next, to say why sulphuric acid was the poorest digester (and produced the least swelling) while hydrochloric was the best (even when employed in the same ionic concentration) I naturally concluded that the SO_4 radical had to be responsible. The addition of a neutral salt containing SO_4 to a hydrochloric acid should therefore not only reduce the proteolysis but also the swelling. When experiment verified this conclusion I had discovered an *antagonism between acids and neutral salts* in a physico-chemical system of known composition, which at once made clear many a similar physiological antagonism. This experiment pointed the way for the overcoming of the last difficulties in the establishment of the colloid-chemical notion of water absorption; for, in the swelling action of acids upon proteins and in the effects of neutral salts in the reduction of this swelling, there appeared at once not only all the possibilities for explaining that behavior of living cells which had previously not fitted in with the laws of osmotic pressure, but all those phenomena as well which had formerly been accepted as proof for the tenability of the osmotic concept. I say this because I had not originally anticipated such a result. I had expected only to find something which would modify or add itself to the osmotic concept of water absorption. The experimental findings on colloids relegated it to a secondary place, and, in my opinion, the osmotic notion has today lost all significance for the problem of water absorption and secretion (as well as the absorption and secretion of dissolved substances) by normal living protoplasm.[1]

Upon what, now, does the colloid-chemical theory of water absorption by protoplasm rest? It rests upon *the qualitative and quantitative analogy between the laws which govern the taking up and giving off of water by any simple protein colloid (like fibrin, gelatin, aleuronat, wheat protein, etc.) and the laws which govern the absorption and secretion of water by any cell, tissue, organ or organism.* These laws as established for fibrin, for example, may be summarized as follows:

1. Fibrin absorbs more water in any solution of acid or alkali than in pure water, the amount of such absorption increasing progressively, up to a certain point, with every increase in the concentration of the acid or alkali.

2. The addition of any salt, even a neutral salt, to such acid or alkaline solution reduces the amount of the swelling and this (a) according to the concentration of the salt added and (b) its kind. Comparative experiments show that the acid radicals follow the order: chloride, bromide, iodide, acetate, sulphate, phosphate, citrate; while the alkaline radicals succeed each other as NH_4, K, Na, Li, Mg, Ca, Sr, Fe, Cu, Hg, when the least powerful dehydrator is, in each instance, mentioned first.

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[1] The one place where in *pathology* it may, in totally modified form, play a rôle I have discussed elsewhere. Martin H. Fischer, "Soaps and Proteins." New York, 1920, p. 246.

3. The taking up and giving off of water is in large measure reversible.

4. Of substances other than the acids or alkalis which increase the water absorbing power of fibrin may be mentioned urea, pyridin and various amines. The increased hydration capacity induced by these substances is, however, different from that produced by acids or alkalis, for it is *not* markedly reduced
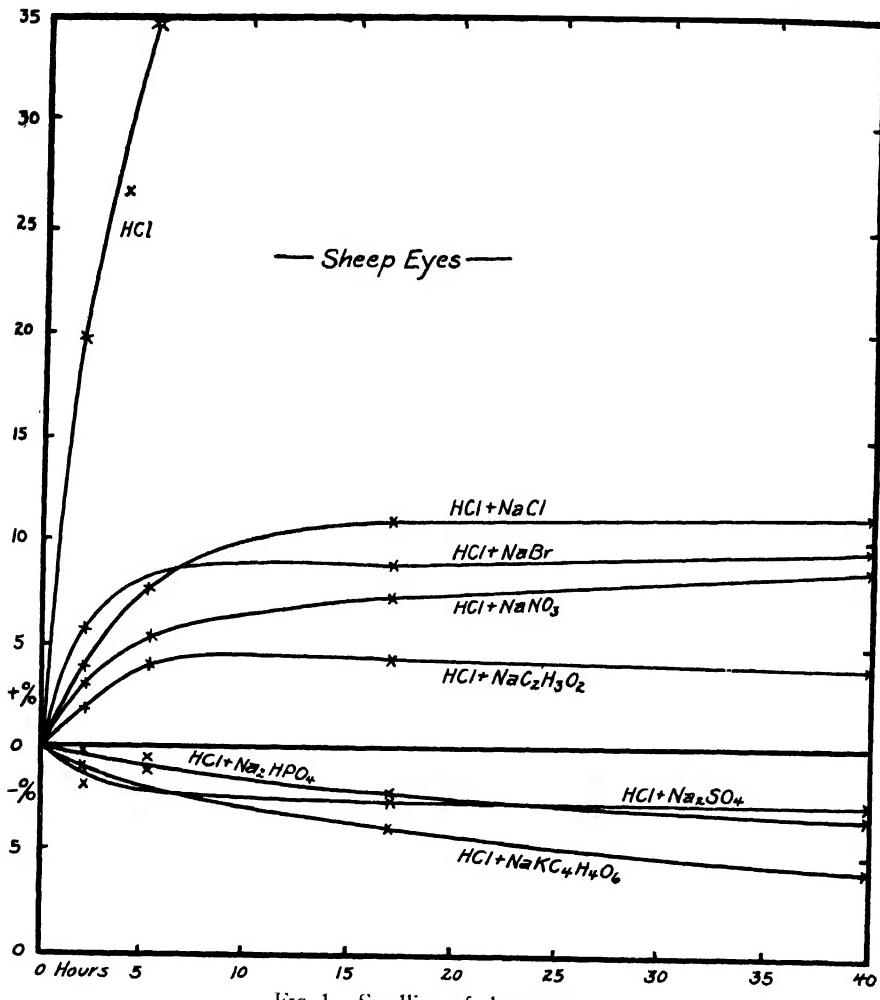
FIG. 1 -- Swelling of sheeps eyes.

through the addition of neutral salts, but is reduced through the presence of the sugars which affect acid or alkali swelling relatively little.

An entirely similar set of laws may now be established not only for every other protein (gelatin, gluten, aleuronat) but, what concerns us more, for every cell, tissue, organ or organism. The isolated cells of the blood, tissues like muscle, brain or spinal cord, and whole organs, like the eye, behave, in this respect, exactly like granules of fibrin or masses of gelatin. A single

set of swelling curves as established for the eye may serve to illustrate the parallelism better than many words. In Figure 1, time is plotted on the horizontal and degree of swelling in percentage increase in weight over the original, on the vertical. In a pure acid the eye absorbs enough water to burst—an experiment which showed for the first time how "glaucoma" could be produced at will, and of an intensity never observed clinically. The addition of any neutral salt reduces this swelling, the order of the salts being that observed when pure proteins are studied. In this antagonism between acids and neutral salts is given the foundation for a proper therapy of this disastrous disease.

It is sometimes said that while colloid swelling and water absorption and secretion in living tissues thus parallel each other qualitatively, colloid water absorption is nevertheless inadequate to explain the higher grades of water holding power shown by tissues in edema. A high grade of clinical edema is represented by a thirty per cent increase in body weight. It has already been pointed out that no *osmotic* pressures are available to explain such. But experimentally I have made muscle or brain substance take up two hundred and fifty per cent its original weight in water. Colloid swelling readily accounts for even such extremes. Taking one quarter of normal tissue as its dry weight we have it holding but three times this weight physiologically or fourteen times this weight in the extreme instance mentioned. How easy to explain these figures when it is remembered that simple protein colloids like gelatin or fibrin take up twenty to thirty times their weight of water under comparable circumstances, while other colloids (like various soaps which have so much in common with the protein derivatives) absorb even ninety-nine times their weight in water and still remain solid!

IV. WATER ABSORPTION AND SECRETION IN THE COMPLEX ORGANISM

In what has been said we have seen how a fibrin flake floating in a solution of any kind swells and shrinks as does any isolated cell (an ameba) or group of cells (a tissue, like muscle) in their milieu of similar composition. A better test of the colloid concept of water absorption is, however, presented when we try its virtues in the analysis of water absorption and secretion in the complex system which we term the living mammal. At first sight the latter problem seems very different from the former, for while a single cell like the ameba takes up or gives off water "from all sides" we have, in the complex organism, whole organs which are seemingly set apart for absorption (the large intestine, for example) or secretion (the kidney) alone. But this is only a matter of point of view, for the intestinal wall absorbs only as long as we look at it from the side of the gut; it secretes when viewed from the side of the blood. And a kidney which usually only secretes water, can, under proper circumstances, be made to absorb this from the kidney pelvis. What really characterizes absorption and secretion in the higher animals is therefore the fact that, *from the point of view of the organism as a whole, it occurs predominantly in one direction.*

Every *absorbing system* consists of three phases: (1) water plus various dissolved substances; (2) an absorbing membrane, a gel; and (3) an absorbing liquid, as blood or lymph, a liquid colloid solution. It is of greatest importance to distinguish between the absorption of water, on the one hand, and the absorption of dissolved substances, on the other. Only at times do

these occur in the same direction and frequently they take place in opposite directions.

In the study of the absorption of fluid from the peritoneal cavity we find first, that pure water is absorbed most rapidly, that this water is absorbed more slowly when salt is present in it, and increasingly so with every increase in the concentration of the salt. If the absorption of water from equimolar salt solutions is compared, the Hofmeister series for the bases and acid radicals is followed.

Similar facts hold for the absorption of water from the lumen of the intestine.

Water absorption occurs best from the large intestine, less well from the small intestine and scarcely at all from the stomach. This series corresponds with the fact that the most venous blood (in other words the blood highest in carbonic acid and poorest in oxygen) is found in the blood vessels of the large intestine, a less venous blood in the blood vessels of the small intestine and an exclusively arterial blood in the blood vessels of the stomach. Colloid-chemically expressed this means, however, that the blood richest in carbonic acid is the best absorber, for it represents a colloid incompletely hydrated. Such blood abstracts water from the gel absorbing membrane which, in its turn, becoming unsaturated, absorbs water from the lumen of the intestine when it is present. If a salt solution lies in the gut instead of pure water, the salt antagonizes the hydration of the membrane and, depending upon its concentration and kind, therefore interferes with the absorption of water. If the concentration is sufficiently high or when salts possessed of certain radicals are used (as the cathartic salts or calomel) an actual reversal of the water absorption may occur, in other words, water be secreted into the intestinal lumen.

Every *secretion system* is also composed of three phases and here again we must distinguish between the secretion of water and the secretion of substances dissolved in the water. Water secretion is possible only when arterial blood is furnished an active gland, in other words, a blood low in carbonic acid, high in oxygen and containing in addition "free" water. The intravenous injection of no amount of "bound" water, as in the form of blood, blood serum or of a "solution" of gelatin, casein or gum arabic increases, in the slightest, any secretion. But if the water is injected free, as in the form of a physiological salt solution, an increased urinary flow follows at once. By merely increasing the concentration of the salt the urinary output may be heightened to two or three times the amount of injected fluid, while when equal volumes of equally concentrated solutions of different salts are injected the diuretic effect again follows the Hofmeister series.

It is of importance for the colloid-chemical theory of secretion to note that the so-called diuretic effect of any salt is not dependent, primarily, upon a local kidney action but upon a general effect of the salt upon all the tissues of the body. The diuretic effect of a salt solution is the algebraic sum of the free water injected plus the amount set free through the action of the salt.

So far as the mechanism of secretion by the kidney (or any other gland) is concerned let us recall that variations in the amount of water secreted do *not* parallel any variations in the amount of oxygen consumed, a fact which argues strongly that water secretion is purely a filtration process. How closely filtration through a hydrated gel simulates this kidney filtration process may be illustrated as follows. A cup composed of three per cent sodium stearate

does not let a drop of fluid filter through when filled with a liquid hydrated colloid like sodium oleate or gelatin. When, however, these solutions are diluted so that they contain free water, filtration starts. All salt solutions pass through such a cup and increasingly faster with every increase in the concentration of the salt. What is still more interesting is that when equally concentrated solutions of different salts are compared the Hofmeister series (for the bases) is rediscovered.

These remarks on absorption and secretion explain also why blood and lymph remain in their vessels. Blood and lymph are not liquids in which the water is free (as in dilute salt solutions), but are solvated colloids; in other words, liquids in which the water is bound to the colloids. This fact, which I first brought out in 1910,[2] constitutes the basis for the modern therapy of shock in which, instead of the older salt solutions, there are now used as injection fluids, blood, gelatin, acacia, etc., in other words, fluids in which the water is largely bound.

V. The Nature of the Lyophilic Colloid

Having shown that the laws of water absorption and secretion as observed in simple protein colloids parallel qualitatively and quantitatively the laws of water absorption and secretion as observed in any living cell, tissue or organism, we need now to retrace our steps and ask regarding the nature of the hydrophilic (lyophilic or solvated) colloids themselves and the nature of the physico-chemical changes which are involved when we say that a colloid "holds" water (or any other "solvent") or changes the amount of this.

For our biological purposes we may start with the chemical fact that proteins represent complexes of amino acids, in goodly portion complexes of amino fatty acids. As amphoteric substances, these are capable of yielding *at least* two series of compounds with bases or acids. If we strike off the amino group from the amino fatty acids, we have left the simple fatty acids which united with bases, yield the soaps. A study of the colloid-chemical behavior of these, a study for instance of their water absorbing qualities, should yield us at least a part of the laws valid also for protein and protoplasm. If we ask how the different soaps behave colloid-chemically toward water we discover the following:

1. When we examine, say, the sodium soaps of any fatty acid series, it is found that they show a steady increase in water-holding capacity with increase in the complexity of the fatty acid. While a gram-molecule of sodium caprylate holds 200 cc. of water, sodium arachidate holds thirty-seven thousand.*

2. If with a given fatty acid the base is varied the water-holding capacity follows the general order NH_4, K, Na, Mg, Ca, Ba, Hg, in other words, the Hofmeister series.

3. For the general theory of the solvated colloids the fact is of fundamental importance that the same laws, including the Hofmeister series, appear when these colloid soap systems are prepared of anhydrous soaps and non-aqueous and water-free media of the type of hexane, heptane, benzene, xylene, mono-, di- and triatomic alcohols.

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[2] Martin H. Fischer, "Edema," New York, 1910, p. 186; *Kolloidchem. Beihefte,* 2, 304 (1911); *ibid.,* 3, 385 (1912); "Edema and Nephritis," New York, 1915, 2nd ed., p. 333.
* Beyond a certain upper limit, there is a decrease. Regarding this zone of maximum colloidality, see Chapter I, Vol. I, of this series. *J. A.*

One is not accustomed to say that a soap is formed through the "influence" of an alkali upon the fatty acid; it is equally wrong to say that proteins are thus "influenced" by any base (or by acids or salts). *New* soap-like *protein*

SOAP IN WATER

SOAP IN WATER

WATER IN SOAP

Fig. 2-A.

WATER IN SOAP
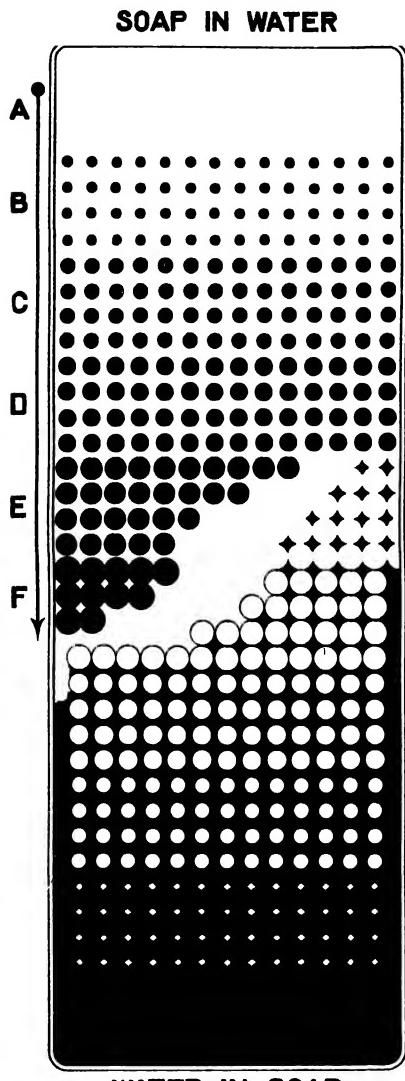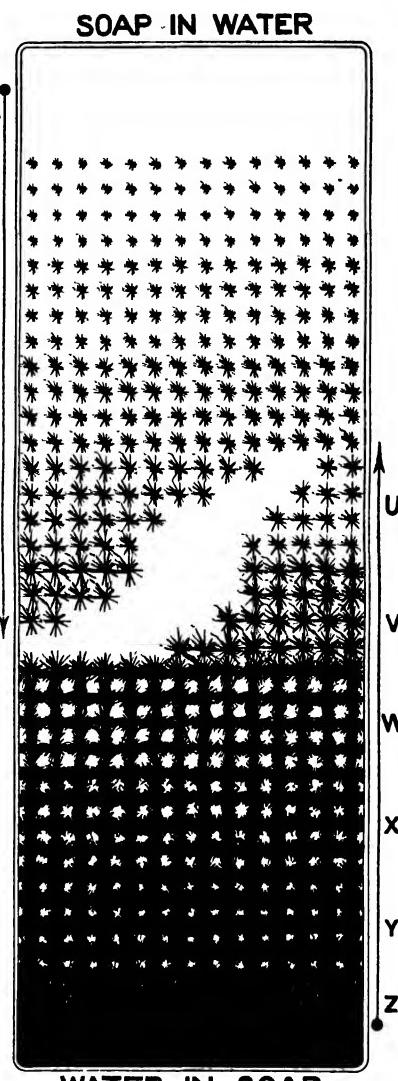
Fig. 2-B.

derivatives are formed with *new* properties, such as different capacities for holding water.

If now we try to say what happens when any soap yields a gel with any given dispersion medium (any so-called "solvent"), the following facts are of interest. At 100° C. a 25 per cent mixture of potassium palmitate with

water or ethyl alcohol shows all the properties of an ordinary molecular solution. When the mixture is cooled to room temperature it first becomes opalescent, progressively more viscid and finally yields a fairly solid gel.

What, I believe, happens not only in this illustration but in all lyophilic colloid systems, is represented by the diagrams *A* and *B* of Figure 2. Diagram *A* is representative of systems in which the two phases are liquid at the temperatures employed, diagram *B* when the separating phase is solid or crystalline. If, for illustration, the system soap/water is chosen (say, potassium oleate/water in illustration of diagram *A* and sodium stearate/water in illustration of diagram *B*) the entire set of systems illustrated in the two diagrams may be obtained through mere change in temperature. At higher temperatures, the soap "dissolves" *in* the water and there results a "true" solution. This matter is represented by the region marked *A* in the diagrams (the soap is dispersed molecularly or ionically *in* the solvent). As the temperature is *lowered,* the solubility of the soap in the water is decreased and as the saturation point for the lower temperature is attained. the soap particles assume more than molecular size. By definition, therefore, we approach with falling temperature the realm of the colloids, or that of dispersions of one material in a second with the degree of dispersion showing dimensions greater than the molecular. This gradual increase in the size of the soap particles (or increase in their number) with lowering of the temperature is represented by the regions *B, C, D, E* and *F*.

Such supersaturation with agglomeration of particles, while yielding us a colloid system, does not yet tell us whether such will be lyophobic or lyophilic (or, depending upon the solid or liquid nature of the separating phase, a suspension or an emulsion colloid). *The lyophobic colloid results when the solvent is not soluble, the lyophilic when the solvent is soluble in the precipitating phase.* When soap falls out of solution from such a solvent as allyl alcohol, the former of these possibilities is satisfied (and we get a lyophobic colloid); when it falls out from most other alcohols or, as in our illustration, from water, the latter is satisfied (and we get a lyophilic "sol" or "gel"). The black circles or crystal clusters in the diagrams of Figure 2 represent more, therefore, in the latter instance than precipitates of pure soap; they are this, plus a certain amount of the water (or other "solvent") dissolved in them.

At a sufficiently low temperature the soap aggregates will have become so large or so numerous as to touch and coalesce. This process continued sufficiently must yield ultimately a single system in which the soap has now become the "solvent" *for* the water. Diagrammatically this situation is represented by the zone *Z* of Figure 2.

Between the upper extreme *A* of a solution of the soap in the solvent and the lower extreme *Z* of the solvent in the soap, there exist two main zones of mixed systems,—one below the upper (*B, C, D* and *E*) consisting of a dispersion of solvated-soap in the soaped-solvent, and a second above the lower (*Y, X, W* and *V*) consisting of soaped-solvent in the solvated-soap. These two mixed system (if the soap is liquid) are in essence emulsions, but of opposite type and as such (even when of the same *quantitative* chemical constitution) are possessed of totally different physical properties. The former corresponds, for example, to an emulsion of oil-in-water, the second to one of water-in-oil, and as the former (as illustrated by milk) will mix with water, wet paper and show a certain viscosity value, the latter (as illustrated

by butter) will mix only with oil, will grease paper and show an entirely different viscosity.[3]

Returning to the lyophilic soap and the diagrams, it is obvious that as we descend, with lowering of temperature, from the region *A*, we pass in the regions *B, C* and *D* through increasingly viscid liquid colloid "solutions" (so-called sols) but all of them emulsions of the type solvated-soap in soap-water. In the region *E*, the particles of solvated soap almost touch and here the highest (liquid) viscosity is obtained. In *F* they do touch and now form a continuous external phase. At this point we change to the opposite type of emulsion (to one of soap-water in solvated-soap) and the previously liquid colloid becomes solid. As ordinarily put, the mixture gels.

It is of interest next to emphasize how this concept of the changes which a soap/water system suffers in passing from a liquid sol to a dry gel may help to explain some of the "strange" characteristics of colloid systems.

It is clear, first, that this concept of the lyophilic colloid sets no limitations upon the nature of the materials that may make up such a system and makes no specifications as to the nature of the forces which guarantee its stability. They are, in general, any or all the forces which appear or are operative whenever "solution" of any kind occurs. This is emphasized because there has been much written, for example, regarding the all-important effects of such single elements as the electrical charges, the hydrogen ion concentration, etc., in determining the stability of colloids in general or that of the lyophilic colloids in particular. We do not wish to deny that electrical charges or hydrogen or hydroxyl ions may sometimes play some rôle in determining the behavior of some colloid systems, but this is an altogether too narrow view to take of the lyophilic colloids in general. Electrical forces may be apparent, for example, in systems composed of soaps and water or of proteins and water; they have to be whenever the system as a whole has any of the phase, soap- or protein-dissolved-in-water present in it, in other words in all the regions of our diagrams below the level *Y*. But the discoverable electrical charges or the hydrogen or hydroxyl ion concentrations no more determine the properties of such colloid systems than the H or OH ions of distilled water explain its physical state; the electrical charges and the hydrogen and hydroxyl ion concentrations are only the accidental consequences of the fact that one of the phases of the colloid system (soap or protein) is "soluble," hydrolyzable and ionizable in any excess of water that may be present. That the electrical phenomena are an accidental consequence, and not a cause of the behavior of such colloid system, is proved by the fact that soaps form as good or better colloid systems with the most varied types of "organic" solvents (as the anhydrous alcohols, toluene, benzene, chloroform or ether). And where are the electrical forces when lyophilic colloid systems are built up of nitrocellulose with ether and alcohol, agar-agar with water, or rubber with benzene? What remains are two mutually soluble substances and the forces active are any or all that appear whenever such "solution" occurs.

The diagrams serve to clarify also the colloid-chemical concepts of *hysteresis, gelation capacity, swelling and syneresis.*

When it is borne in mind that the absolute solubility values of any two mutually soluble substances are rarely the same and that the rates at which they go into solution in each other are usually different, it becomes apparent

[3] See in this connection Martin H. Fischer and Marian O. Hooker, *Science*, **43**, 468 (1916); *Kolloid-Z.*, **18**, 129 (1916), *ibid*, **18**, 242 (1916); "Fats and Fatty Degeneration," New York, 1917, p. 20.

why, with lowering of temperature for example, a lyophilic colloid system will tend, in general, to set at a temperature lower than that at which it will liquefy when, on a reversal of experimental conditions, the temperature is raised.* The point at which a lyophilic colloid system gels is obviously that at which the solvated colloid phase becomes the external one. The colloid system at this point still contains, as an internal phase, a solution of the colloid in the solvent. Gelation capacity is therefore always greater than the solvation or hydration capacity of a colloid. The latter measures the solubility of the solvent in the colloid material. The increase in the volume of the latter as the solvent is taken up is the measure of its ability to "swell." The zone *Z* in the diagrams covers the swelling capacity of a given material with its "solvent"; the gelation capacity embraces all the zones above this up to and including the zone *V*. As soon as this zone is passed, the external hydrated colloid phase may not be adequate to inclose all the solution of colloid-in-solvent, at which point the system as a whole tends to sweat, in other words, to show the characteristic phenomenon of syneresis. The failure to inclose adequately the internal phase will be more likely if one of the materials of the mutually soluble system is solid than when both are liquid, wherefore colloids of the hydrated solid type (like sodium stearate/water, silicic acid/water) will show a greater degree of syneresis than more liquid ones (like sodium oleate/water, rubber/benzene, etc).

VI. Mutually Soluble Systems

As soon as we have said that protoplasmic behavior (like its power to absorb or secrete water) is comparable to the behavior of hydrated protein, that the latter in its water absorption and secretion is identical with the hydration and dehydration of simple soaps, and that, finally, the soaps with their "solvent" are merely illustrations of mutually soluble systems, we become interested in the classic example of the latter, namely that of phenol with water, to the significant behavior of which for the theory of the solvated colloids Wolfgang Ostwald[4] called attention many years ago.

We purpose now to investigate the system phenol/water which in the two phases, water-dissolved-in-phenol and phenol-dissolved-in-water, gives us in handy laboratory fashion the analogues, respectively, of the zones *Z* and the zones *A* of the diagrams of Figure 2. *Protoplasm is comparable to the solution of the water in the phenol; the secretions from the body, to the solution of the phenol in the water.* The following paragraphs concern themselves chiefly with the electrical conductivities of these two phases, more especially that of the water-dissolved-in-phenol. The significance of these findings for an understanding of some of the electrical phenomena observed in protoplasm is returned to later.

Our standard phenol/water systems were prepared by measuring 50 cc. of phenol, liquefied at 50° C., into 100 cc. cylinders and adding 50 cc. of water or the various solutions described in the experiments. After thorough mixing, the cylinders were set aside for 18 hours at 22° C. What happens when water only is added is illustrated diagrammatically in the cylinder marked 1 of Figure 3. It will be observed that two solutions are formed, a lower one of phenol containing water (and usually referred to in the succeeding pages as the hy-

* There is, as well a kinetic aspect to this problem. *J. A.*
[4] Wolfgang Ostwald, *Kolloid-Z.*, 1, 335 (1907); "Introduction to Theoretical and Applied Colloid Chemistry," New York, 1922, 2nd ed., p. 95.

drated phenol phase) and an upper one of water containing phenol (and usually referred to as the phenolated water phase). After mutual solution has taken place the two phases are unequal in volume. As water dissolves in the pure phenol its volume increases. The phenol "swells" some 28 to 30 per cent. How this degree of swelling alters under various circumstances and the degree of resistance of the two phases to the passage of an electrical current were the main themes of experimental inquiry.

The electrical resistance of the two phases was measured in the customary fashion with a pair of fixed, platinized platinum electrodes of the dip type, by the ordinary Wheatstone bridge arrangement and a telephone. The same electrodes, having the constant 0.0793, were used in all the experiments, they being repeatedly checked, to show that they had suffered no change, with a 0.02 N potassium chloride solution. Since the same electrodes were used throughout, the resistance values are given as observed without calculation in terms of specific resistance. The phenol used was specially purified. Only such phenol will exhibit the high initial resistances recorded in the following paragraphs. Our phenol when liquefied at a low temperature and then permitted to crystallize about the electrodes at $22°$ C. showed a resistance of more than 210,000 ohms. The purest phenol of the open market showed under similar circumstances a resistance of only 2,000 ohms. Such difference is dependent upon the presence of water,[*] neutral salt, dissolved glass and atmospheric gases in the commercial preparation.

The water employed in our experiments was distilled from silver. Freshly obtained it had a resistance of 100,000 ohms. The nature of our experiments was such, however, that we could not protect our ultimate mixtures from air, contamination with carbon dioxide or the effects of our glass containers. Control experiments showed that these circumstances might cut the electrical resistance of our distilled water to 25,000 ohms.

We can sum up the findings of our experiments in the following categorical statements.

1. The electrical resistance of pure phenol is reduced progressively by every increment of water added to it up to its saturation point. From the initial value of 210,000 ohms under the conditions of our experiment (contamination with air and glass), a final resistance of more than 20,000 ohms is registered.

2. When alkali or acid is added to a phenol/water system, the electrical resistance of the hydrated phenol phase falls progressively with every increase in their concentration in the system. Under otherwise similar conditions, alkali reduces the electrical resistance more than acid. From the enormous initial resistance (more than 20,000 ohms) of the pure hydrated phenol the fall is so great between the concentrations of 0 02 to 0.10 N HCl (or NaOH) that the ultimate values attained are measurable in two or three hundred for the acid and less than one hundred for the alkali. The resistance of the water phases in equilibrium with these phenol phases is less than 10 ohms in the case of the acid and less than 100 ohms in the case of the alkali.

While both acid and alkali reduce the resistance of hydrated phenol, only the latter markedly influences its volume. While acid seems to decrease it slightly, alkali leads to a progressive increase in the volume of the phenol

cell was, according to their notion, a sacule of fluid encompassed by a semi-permeable membrane, a membrane, in other words, impermeable to all dissolved substances but readily permeable to water. A first observation to disturb this concept of the cell, was made by these authors themselves, when they noted that various dissolved substances would diffuse into cells and precipitate the tannin contained therein. This fact with many others touched upon above [11] led us early to deny the existence of semipermeable membranes about cells.[12] How now are we to understand these biological studies of "permeability" which have so long been interpreted through the assumption that some kind of a membrane ("semipermeable," "lipoid" or "plasmatic") exists about cells after we have done away with this? The answer is simple.

We have repeatedly emphasized that the hydrophilic colloid (especially one protein in nature, like a particle of fibrin) is possessed of all the powers of taking up and giving off water shown by the living cell while it has, at the same time, all the powers of taking up and giving off dissolved substances which are characteristic of living matter.[14] All this occurs, of course, without the need of assuming that the hydrated colloid mass has a membrane about it which differs in any way from the rest of the fibrin flake. Our recent studies [14] have shown that such a protein mass, combined with acids and alkalis or salts and saturated with water (and comparable in this form with the foundation material constituting the cell) is in essence a system comparable to water-dissolved-in-phenol. It is this water-dissolved-in-phenol system which when subjected to the action of alkalis or of salts "swells" and "shrinks," shows in other words the biological phenomena of plasmoptysis and plasmolysis (too commonly still explained on an "osmotic" basis) just as does a hydrophilic colloid (a protein) or a living cell. But this system shows also the "strange" phenomena of "permeability" to dissolved substances so characteristic of living matter. When phenolated water and hydrated phenol are in contact with each other, the latter phase will take up certain substances better than the former or vice versa. And here the analogy to what has been observed in permeability studies on living cells is again great. The hydrated phenol is quickly "permeable" to the most varied dyes (Nile blue sulfate, neutral red, methyl red, methyl violet, methyl green) and will practically exhaust the water phase in a few hours. Other substances (like eosin or iodine) will pass in less quickly and less completely. All the salts (so often held by various biological students incapable of entering or leaving the uninjured living cell) enter the hydrated phenol phase either very slowly or not at all. Ferric chloride, cupric acetate, cerium sulfate (or their hydrolytic products) all enter the phenol phase but with decreasing facility; chromium chloride, chromium sulfate, cobaltous chloride, nickel chloride seem to remain entirely in the aqueous phase. Of "colloid" substances, infusorial earth concentrates in the aqueous phase, boneblack in the phenol phase.

2. The electrical experiments described above were devised to show, if possible, that the peculiarities which living matter exhibits to the passage of an electric current through it may also be readily understood as soon as it is remembered that the cell is not, as so long conceived, a dilute solution of x-in-water but one of water-in-x, comparable to the system water-in-phenol.

[11] *Vide* supra.
[12] Martin H. Fischer, *Am. J. Physiol.*, **20**, 330 (1907); *Pflüger's Arch.*, **124**, 69 (1908); *ibid.*, **125**, 99 (1908); *ibid.*, **127**, 1 (1909), *ibid.* **127**, 46 (1909); "Edema," New York, 1910
[13] Martin H. Fischer, "Edema," 200, New York, 1910, "Edema and Nephritis," 3d ed., 206, 318, 367, 640, New York, 1920.
[14] Martin H. Fischer, *Science*, **48**, 143 (1918); "Soaps and Proteins," New York, 1921, p. 205.

The first fact which strikes the student investigating the electrical resistance of cells or biological fluids is its height.[15] In spite of the conclusion that a "physiological" salt solution (say a 0.7% or 0.9% NaCl) is supposed to be "osmotically" comparable with that of the salts "dissolved" within the ordinary living animal cell or its fluid, the former will register, with a standard pair of electrodes, only 1/5 to 1/35 the electrical resistance of uninjured cells, muscle juice, lymph, blood, egg white or egg yolk. This old biological truth can be understood only by denying to the salts found in protoplasm any large existence in uncombined form [16] or by concluding that the cell is a different sort of solvent for these salts than is water. Experimental facts, we think, support both these conclusions. The high electrical resistance characteristic of living protoplasm cannot be observed in solutions of the type phenol-dissolved-in-water but in those of the type water-dissolved-in-phenol.

When it comes to the changes in electrical resistance evidenced by living cells when subjected to intoxication, injury or environmental change, the following findings are noted by all observers. Through the action of acids or alkalis, the electrical resistance of protoplasm is reduced. In similar fashion acids and alkalis are most powerful in decreasing the electrical resistance of hydrated phenol. But potassium hydroxide affects protoplasm more than equally concentrated sodium hydroxide, and this more than calcium hydroxide. Hydrated phenol behaves similarly. Solutions of single salts also reduce the electrical resistance of living cells but are less effective in this regard than the acids or alkalis. This is characteristic, too, of hydrated phenol. A single salt when acting upon a cell may, however, show a lack of uniformity in this regard, at certain concentrations proving more effective than at others. This, too, is seen in the curves illustrating the behavior of hydrated phenol. The absolute differences in physiological effect exhibited by different salts when employed in comparable concentrations is also repeated in the case of hydrated phenol. Finally, the physiological antagonism between different salts (the ability of a divalent radical, for example, to counteract the reducing effects of a univalent one) may also be observed upon hydrated phenol. Even the effects of certain non-electrolytes in reducing the electrical resistance of protoplasm (as that of the anesthetic alcohols) may be rediscovered in hydrated phenol systems.

What has been said for phenol/water systems is true of many other mutually soluble systems. Quinoline and aniline, for example, behave much like phenol [17] and what has been said of these substances holds also for the lower fatty acids, the soaps and the various proteins.

Returning, now, to the hydrated colloids we discover that the electrical behavior of a *gelatinized* colloid is comparable, *not* to the phase phenolated water but to that of hydrated phenol. When a soap/water system or a protein/water system is cooled, the electrical resistance curve shows an abrupt

[15] See the many observations covering this point beginning with W. Roth, *Zentr. Physiol.*, 11, 271 (1897); Bugarszky and Tangl, *ibid.*, 11, 297 (1897); *Pflüger's Arch.*, 72, 531 (1898); G. N. Stewart, *Zentr. Physiol.*, 11, 332 (1897); *J Physiol.*, 24, 356 (1899); *Am. J. Physiol.*, 49, 233 (1919).

[16] See in this connection Martin H Fischer, "Soaps and Proteins," New York, 1921, p. 228.

[17] It seems to hold also for the system $SO_3/H_2SO_4/H_2O$. The electrical resistance of SO_3 is very high, falling as H_2O is added After passing through a low point, the resistance rises to fall a second time. From the second low point further addition of water leads to the ordinary increase in resistance characteristic of increasingly dilute solutions of sulphuric acid. We think that the explanation of this well-known curve is about as follows· SO_3 has a high resistance. "Dissolving" H_2SO_4 in this, lowers the resistance, to be followed by an increase as the SO_3 "dissolves" in the H_2SO_4. The "solution" of H_2O in the "concentrated sulphuric acid" *lowers* its resistance, to be followed by an increase as the H_2SO_4 dissolves *in* the water.
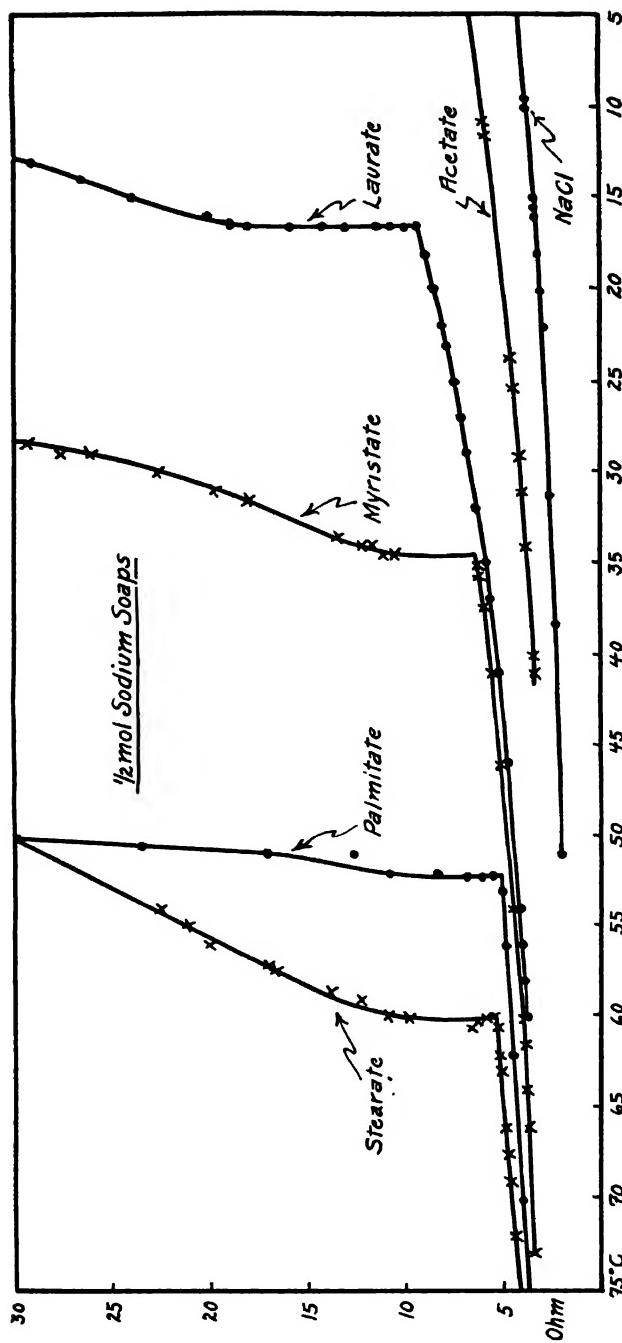
FIG. 4.—Changes in resistance of soaps on gelation.
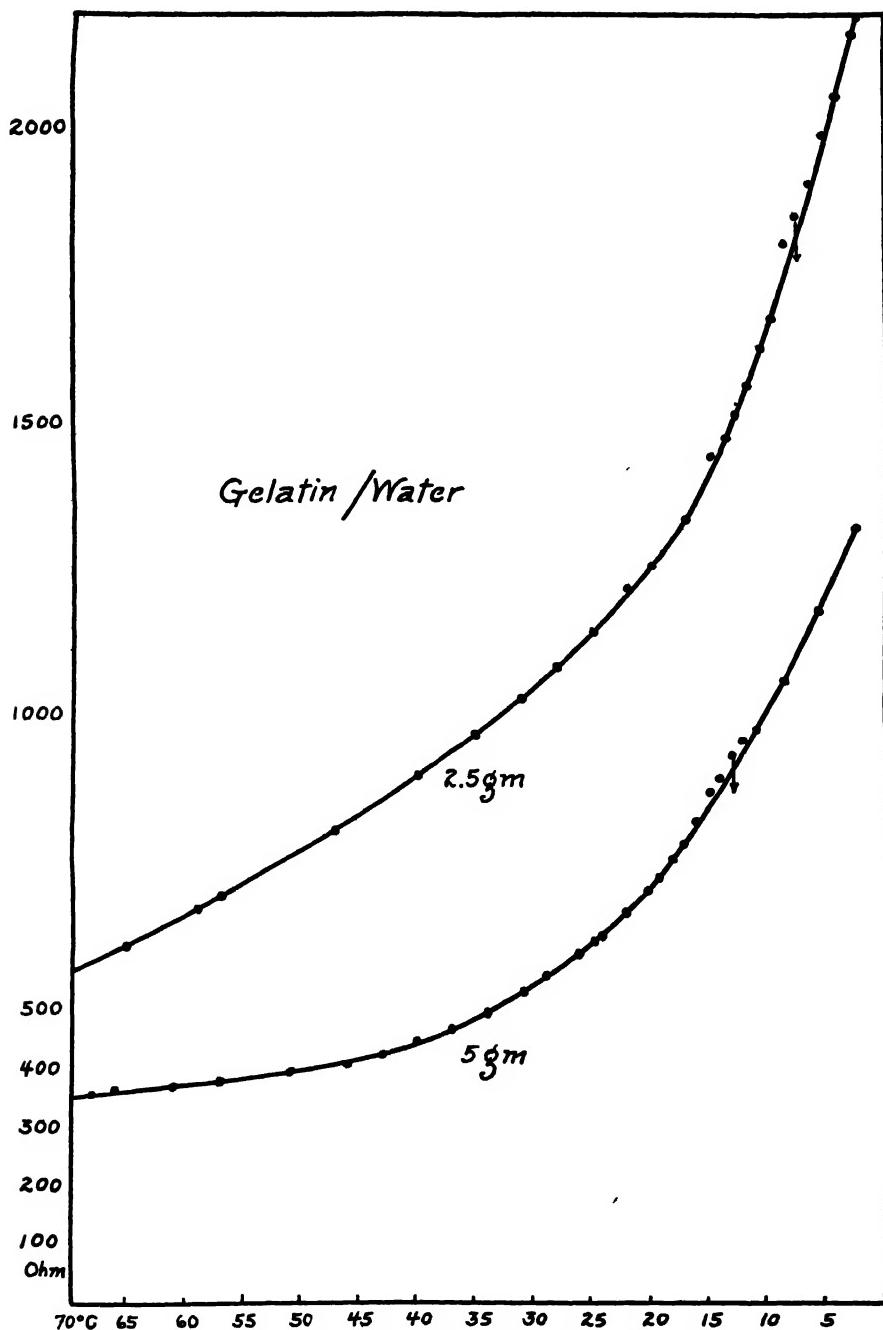
Fig. 5.—Changes in resistance of gelatin on gelation.

turn upwards (it may be right angled) as the gelation point is passed. This is clear proof that in the process of gelation there occurs a fundamental change in structure—a change in our terminology from what is essentially a "solution" of x in the solvent to one of the solvent in x. This is illustrated for several sodium soaps of the acetic series in Figure 4 and for two concentrations of "ashless" gelatin in Figure 5.

VIII. The Physico-chemical Structure of Protoplasm

Bearing in mind the facts regarding mutually soluble systems in general and the solvated colloids in particular what have we to conclude regarding the structure of protoplasm? Protoplasm is in essence not, as generally held, a solution of x-in-water but a reverse type of system, a solution of water-in-x. The excretions .from protoplasm (like the urine, sweat or gastric juice) approximate, on the other hand, the type protoplasmic-substance-dissolved-in-water. As proof may be cited the high electrical resistance of cells and body fluid as compared with the approximately "normal" conductivity exhibited by the secretions. Protoplasm is comparable to the solution of water in phenol, the secretions to the solution of phenol in water.

The bases, acids and salts of protoplasm are chiefly not free, but bound chemically to the proteins. Those which are free are "dissolved," but dissolved not in free water but in a hydrated biocolloid. Proof for this may be brought from various directions. Fresh animal or plant tissues do not taste like the aqueous solutions of the electrolytes extracted from them; and their electrical conductivity is so low that this is not even comparable to the conductivity of these electrolytes dissolved in an equal volume of water.

A final proof that protoplasm and the body fluids, like blood and lymph, are something different from dilute solutions, is offered by their behavior toward indicators. They react not like solutions of the type phenol-in-water, but like those of water-in-phenol. Concentrated mixtures of soap with water (solutions of water-in-soap) are neutral to an indicator like phenolphthalein, while more dilute ones are intensely alkaline. This is true also of acid or alkaline gelatinates or alkaline caseinates and may be demonstrated directly upon blood plasma and tissue juices. It is true even of various highly concentrated acids (solutions, in our terminology, of water in the acid). Concentrated sulfuric acid, for example (water in H_2SO_4 or water in SO_3), or concentrated acetic acid (a limited amount of water in acetic anhydride, for example) are strongly alkaline to indicators of the type of methyl red. Dilute solutions of either in water are intensely acid.

The physico-chemical or colloid-chemical analysis of protoplasm will make progress as soon as it ceases its still too prevalent effort to apply the dilute solution laws, including the laws of osmotic pressure, to biological phenomena by force. A better and more fruitful period will be upon us when attention is fixed upon the behavior of what we must call, for lack of a better name, the concentrated solutions, viewing these from a colloid or dispersoid-chemical point of view. When we have discovered their laws, when we have familiarized ourselves with the physico-chemical and colloid-chemical behavior of systems of the type water-dissolved-in-phenol, we shall find ourselves possessed also of the laws which govern the behavior of protoplasm under physiological and pathological circumstances.

The Combination of Proteins with Acids and Bases, with Some Observations on the Origins of Viscosity in Protein Solutions

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1. The Nature of the Forces which Accomplish the Neutralization of Acids or Bases by Proteins.

It is now admitted by all observers who have directed adequate attention to this question, that the proteins accomplish the neutralization of acids and bases in stoichiometrical, that is, molecular or equivalent-molecular proportions [11, 12, 13, 14, 39, 44, 59, 63, 70, 87, 88, 90, 91, 102, 105, 106, 111, 122, 133].* Opinions, however, still differ very greatly as to the nature of the mechanisms by which this neutralization is achieved. A considerable proportion of investigators are reluctant to admit a purely chemical mechanism of neutralization and prefer to invoke a physical mechanism, that is, molecular attraction between the colloidal particles of protein and the molecules of acid or base which are neutralized. Many of these authors appear to regard the chemical constitution of the protein molecule, its synthesis from multiple amino-acid radicals, as a virtually negligible factor in the determination of its behavior. We will not enter into this discussion at length, because experience has shown that it hinges chiefly upon questions of definition. As long ago as 1907 the author ventured to put forward the suggestion that molecular attractions might frequently prove, in ultimate analysis, to be determined by latent atomic affinities [103].[1] This suggestion was rejected by those authors who, at that time, saw fit to comment upon it, but the recent investigations of Harkins [45, 46, 47] and Langmuir [65, 66, 67] have since familiarized us with the idea and have, moreover, established it upon a broad basis of experimental observation which formerly was lacking. Thus it has been shown that the orientation of molecules at the surface of a liquid is determined by their chemical affinity for the molecules which underlie them. It follows that "adsorption" of dissolved substances by surfaces may itself be the expression of chemical affinities, while, in any case, the effects of such "adsorption" are evidently indistinguishable, in the case of the proteins, from the effects of chemical combination.[2]

Furthermore, the impression that dissolved proteins furnish surfaces at which "adsorption" of substances from the surrounding medium may occur, arises from our knowledge of the colloidal character of these solutions, that

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* These numbers refer to references given at the end of this paper.
[1] The author was, however, anticipated by many others in the expression of this opinion. The earliest of these suggestions quoted by Alexander [4] appears to have been advanced by Prescott.
[2] Thus Alexander urges that "the Donnan equilibrium and its consequences which J. Loeb relies upon to prove the formation of definite chemical compounds, are just as well explainable on the basis of a kinetically balanced adsorption."

is, the indiffusibility or extremely slight diffusibility of proteins in solution. From this we infer that the ultimate particles of protein in solution are very large in comparison with molecules of crystalloidal substances, and such particles, it is supposed, must present a surface to the surrounding medium at which adsorption might conceivably occur. But if, instead of confining our attention to these physical properties of the proteins, we regard their chemical constitution, then a number of considerations unite to show that the ultimate *chemical* unit of protein, the molecule itself, that is, must also be very large. Thus the proportion of iron in hemoglobin indicates a molecular weight of 16,000; the proportions of tyrosine, glutamic acid and cystine in casein indicate a molecular weight of from 4000 to 4400; the proportion of phenylalanine in gelatin [81, p. 187] indicates a molecular weight of 11,800. This being the case it is a mere matter of arithmetic to show that the colloidal properties of proteins in solution must follow naturally from the magnitude of their molecules, so that even molecularly dispersed solutions might be expected to display just those colloidal properties which protein solutions do actually exhibit.

There is, in fact, no necessity to invoke aggregation of molecules to explain the low order of diffusibility of protein solutions, although it unquestionably underlies the phenomena of coagulation and gelation which they also display under special conditions. In many solutions of protein it has been demonstrated by the employment of the differential osmometer [3] and by other methods that aggregation does not occur, although in other cases the existence of aggregates of two or possibly more protein molecules may be suggested by these measurements. But if, in many instances, if not in all, single or even double molecules suffice to account for the colloidal behavior of protein solutions, how, then, shall we now regard the surface which separates the protein from the solvent? It becomes the surface of a molecule itself. We do not speak of adsorption at the surface of sodium chloride molecules or water molecules or sugar molecules. This is not to say that it does not occur, of course, but if it did would its effects be in any way distinguishable from those of chemical combination? Then shall we name one and the same phenomenon chemical combination when it involves a small molecule and adsorption when it involves a large one? We may, if we prefer to do so, but clearly in that event we run some danger of establishing a distinction without a difference or, in other words, transferring the whole question to the sphere of definition.*

2. The Inadequacy of Free Amino and Carboxyl Groups to Accomplish the Neutralization of Acids and Bases by Proteins

Reverting to the purely chemical hypotheses which profess to interpret the neutralization of acids and bases by proteins, these are of two kinds. The earliest and simplest view and that which is still upheld by very many who have not yet encountered or devoted sufficient thought to the facts which render

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[3] The employment of a membrane permeable to crystalloids but impermeable to colloids for the measurement of colloidal osmotic pressures, originally suggested by Starling [123, 124] and utilized by Reid in investigating the osmotic pressure of hemoglobin [99] and by Lillie in investigating the osmotic pressure of gelatin [77], is subject to an error due to the displacement of the distribution of diffusible electrolytes on either side of the membrane; the "Donnan effect." This, however, tends to *diminish* the observed osmotic pressure and the maximum diminution is one-half of the actual osmotic pressure of the colloid [72]. If, therefore, the differential osmometer indicates an aggregation of two molecules, the molecules within the membrane may actually be single, but they cannot be aggregates larger than those inferred from the observed osmotic pressures.

* This argument applies to the interfacial layer, but if we consider the colloidal particle or micell *as a unitary whole*, then stoichiometric ratios do *not* apply. See also paper by Wolfgang Pauli, this volume. Some further views of the Editor are given at the end of this chapter. *J. A.*

it untenable, is that the neutralization is accomplished exclusively by the terminal carboxyl and amino groups of the protein molecule. Thus it is common knowledge that the various amino acids are capable of neutralizing either acids or bases in apparent accordance with the equations:

$$R < \begin{array}{c} NH_2 \\ COOH \end{array} + HCl = R < \begin{array}{c} NH_3Cl \\ COOH \end{array}$$

and

$$R < \begin{array}{c} NH_2 \\ COOH \end{array} + NaOH = R < \begin{array}{c} NH_2 \\ COONa \end{array} + H_2O$$
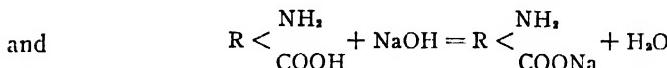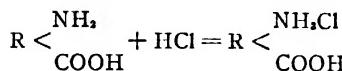
Now the proteins are generally regarded as polyamino acids, built up, as Hofmeister originally suggested [50], by a series of reactions of the type:

$$H_2NR_1COOH + HHNR_2COOH = H_2NR_1COHNR_2COOH + H_2O$$
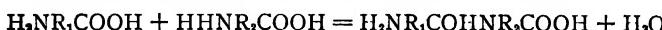
the resultant compound being still an amino acid. This theory is frequently considered to have been substantiated by the synthesis of polypeptides by Emil Fischer [31], since these syntheses admit of representation by formulas of the above type, although, of course, the equations which represent a synthesis do not necessarily define the internal structure of the resultant molecule. The presence of the terminal amino and carboxyl groups in this molecule is thought to account for the amphoteric properties of the proteins, while the multiple combining-capacities of proteins are referred to the presence of diamino or dicarboxyl radicals or of branches in the polyamino acid chain, leading to the presence of a number of terminal amino and carboxyl groups.

To this view, which is still held by very many, the author also formerly inclined [104], but subsequent fuller acquaintance with the quantitative aspects of the neutralization of acids and bases by proteins compelled its abandonment [109]. The facts which render this conception untenable are, in brief, the following:

On treatment with nitrous acid, amino groups react in the manner indicated by the equation:

$$RNH_2 + HNO_2 = ROH + H_2O + N_2$$
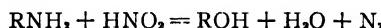
with the liberation of a molecule of nitrogen for each amino group which is decomposed. Utilizing this reaction Levites [75, 76], Kossel [61] and D. D. Van Slyke have shown that only a very small proportion of the nitrogen in protein is present therein in the form of free amino groups. The following are results obtained by Van Slyke and Birchard [130]:

Percentage of Total Nitrogen Present in Free Amino Groups.

Hemoglobin	6.0	Gliadin	1.1
Casein	5.5	Zein	0.0
Hemocyanin	4.3	Heteroalbumose	8.1
Gelatin	3.1	Protoalbumose	9.9
Edestin	1.8		

On the other hand, edestin, after complete hydrolysis by hydrochloric acid, yields a volume of free nitrogen, on treatment with nitrous acid, corresponding to no less than 79 per cent of its total nitrogen content [126]. The investigations of Kossel and Gavrilov, and of Van Slyke and Birchard, have in fact shown that the free amino nitrogen in the unaltered protein molecule exactly

corresponds in quantity to one-half the lysine nitrogen. Hence zein, which contains no lysine [93], yields no free nitrogen on treatment with nitrous acid. The protamin, salmin, similarly contains no lysine and yields no free nitrogen on treatment with nitrous acid [60]. The period required for the complete interaction of proteins with nitrous acid (30 minutes) is longer than that required by the α-amino groups (3 to 4 minutes), but corresponds to that found for the ω-amino group of lysine (126). From these facts Van Slyke and Birchard infer that one of the two amino groups of lysine, namely the ω-group, exists free in the protein molecule and that this group represents, within at most a fraction of a per cent of the total protein nitrogen, the entire amount of the free amino nitrogen determinable in the native proteins by the nitrous acid method. It may, of course, be urged, and has been suggested by some authors [16] that other free amino groups are actually present in the protein molecule but that they are anomalous in that they do not react with nitrous acid. The investigations of E. A. Werner have shown, however, that anomalous amino groups have no real existence [138]. Where they have hitherto been inferred our former conception of the structure of the compounds in which they have been supposed to occur has been shown to be erroneous. The classic instance of urea, which was formerly supposed to contain two amino groups and does not react with pure nitrous acid in aqueous solution, has been shown to arise from the fact that the true structure of urea is not

$$CO < \begin{matrix} NH_2 \\ NH_2 \end{matrix} \qquad \text{but} \qquad HN = C < \begin{matrix} NH_3 \\ O \end{matrix}$$
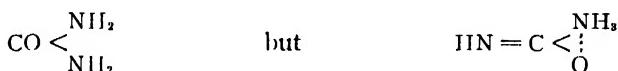
and since it contains no free amino groups it necessarily fails to react with nitrous acid. Similarly, in the native proteins, we may with entire confidence infer that no amino groups exist which do not react with nitrous acid, for the reason that, as Werner has expressed it, "anomalies in such a reaction can have no reality" [138].

We may conclude, then, that only a very small proportion of the nitrogen in proteins is present within their molecules in the form of amino groups. Thus, in the case of edestin, only 1.8 per cent of the total nitrogen is present in the form of free-NH_2 groups.

Now edestin, as Osborne has shown [89] is insoluble in water when uncombined wtih acids or bases. It forms an insoluble hydrochloride containing 14×10^{-5} equivalents of HCl per gram, and, on further addition of acid, passes into solution. Its combining-capacity for acids does not remain constant, however, but continuously increases as the pH decreases, until at neutrality to Tropaeolin, which corresponds to a pH of 2 to 3 [117] it neutralizes 127×10^{-5} equivalents of acid per gram. Hence if the acid were neutralized by amino groups in edestin, the number of these groups in each molecule of edestin must be at least $\frac{127}{14} = 9$. From the former determination it would appear that the molecular weight of edestin is 7000 or a multiple of 7000, and this corresponds with the molecular weight indicated by its tyrosine and glutamic acid content [62] (1 mol. tyrosine + 3 mols. glutamic acid + . . .). Nine amino groups in this molecule would correspond to over ten per cent of the total nitrogen. Since only 1.8 per cent of the total nitrogen of the edestin molecule is actually present therein in the form of amino groups, no less than

eighty per cent of the neutralizing power of edestin for acids must be' accounted for in some other fashion than by the assumption of a union of the acid with free amino groups.

It has been shown by Loeb [81] that one gram of casein suspended in 100 cc. of water is not completely dissolved unless the water contains 6 cc. of 0.1 N hydrochloric acid, or 0.006 equivalents, and that the pH of this mixture, in which the casein is *first* dissolved, is very close to 3, corresponding to 0.001 equivalents of unneutralized acid in 100 cc. The amount of acid neutralized by one gram of casein at the moment that the casein passes into solution is therefore equal to the difference between the amount of acid added to the mixture and the amount which remains unneutralized, that is, 0.005 or 50×10^{-5} equivalents. This must correspond to the formation of a definite compound of hydrochloric acid with casein which, for all we know, may contain one, two, three, or more molecules of HCl combined with one molecule of casein. If we assume that it contains one molecule of hydrochloric acid combined with casein, then casein, at the moment of solution in hydrochloric acid, has a molecular weight of 2000. If the soluble compound contains two molecules of HCl then the molecular weight of casein is 4000. In other words, the molecular weight of casein in acid solution must be some multiple of 2000. According to L. L. and D. D. Van Slyke, casein forms insoluble compounds with acids [134] which would lead us to infer that the higher estimate of the molecular weight is the more probable.

Now when 30 cc. of acid are contained in 100 cc. of 1 per cent casein the pH of the mixture is stated by Loeb to be 1.7. In other words 0.001995 equivalents of acid remain unneutralized, while 0.003000 equivalents have been added. The difference between these figures, as before, indicates the amount of acid neutralized by 1 gram of casein, that is, 0.001015 equivalents, or 101.5×10^{-5}. It is not certain that this represents the maximum combining capacity of casein for acids, but at a pH of 1.55 the combining capacity, similarly calculated, is but slightly increased, namely to 118×10^{-5}. Hence if the first compound, formed when the casein has just passed completely into solution, and containing 50×10^{-5} equivalents of hydrochloric acid per gram, represents the formation of a compound of one molecule of casein with one molecule of hydrochloric acid, then the compound formed in more acid solutions represents a compound of casein with $\dfrac{101.5}{50} = 2$ molecules of hydrochloric acid. If the first compound represented the combination of one molecule of casein with two molecules of hydrochloric acid then the second represents the combination of one molecule of casein with four of hydrochloric acid, and so forth. Thus if the neutralization of the acid is accomplished by amino groups there must be two amino groups in the molecule of casein if its weight is 2000, four if it is 4000, or in other words, one amino group per thousand grams of casein. Now the total nitrogen in casein is 15.7 per cent or 157 grams per thousand. One amino group would correspond to $\dfrac{14}{157} = 9$ per cent of the total nitrogen. But only a little more than half of this amount of amino nitrogen is actually present in casein. At least half the capacity of casein to neutralize acids is therefore attributable to elements in the molecule other than amino groups.

Basing his estimate upon the determinations of Procter and Wilson [97] Loeb infers that one molecule of gelatin is capable of combining with 15 or

a multiple of 15 molecules of a monobasic acid [81, p. 62]. This would correspond, if neutralization were accomplished by means of amino groups, to fifteen amino groups in a molecule weighing 11,800. This would imply the presence of $\frac{210}{11,800}$ or 1.8 per cent of amino nitrogen. The total nitrogen content of gelatin is 18 per cent [4]. Hence $\frac{1.8}{18}$ or 10.0 per cent of the total nitrogen in gelatin must be supposed to be present therein in the form of amino groups, but the actual amino nitrogen content of gelatin is less than one-third of this [130].

Even if we adopt Hitchcock's [49] estimate of the combining weight of gelatin [1120] instead of Procter and Wilson's smaller figure [768] the amino groups required would still be twice as many as gelatin actually contains.

The protamin, salmin, unites with sulfuric acid to form a compound of the composition $C_{30}H_{57}N_{17}O_6$, $2H_2SO_4$, yet salmin yields no nitrogen on treatment with nitrous acid, because it contains no lysine. Sturin [63] contains 67 per cent of its nitrogen in the form of arginine, 10 per cent in the form of histidine, and 6 to 7 per cent in the form of lysine. It yields nitrogen on treatment with nitrous acid corresponding to the ω-amino group of the lysine. Only about three out of every hundred nitrogen atoms in sturin are therefore present in the form of free amino groups. Yet one hundred nitrogen atoms in sturin will neutralize no less than 24 equivalents of acid. Evidently at least twenty of these acid molecules must attach themselves to the molecule of protein at some other points than those provided by amino groups.

The number of carboxyl groups in any protein cannot be much in excess of the number of amino groups, for otherwise the protein would be overwhelmingly acid in character and, besides, since relatively few of the amino acid radicals in most proteins are dicarboxylic acid radicals, if a great excess of free carboxyl groups were present in the molecule, the combined amino groups could not be attached to carboxyl-groups, as they are in the polypeptides, and the splitting of proteins into their constituent amino acids by hydrolysis could not occur. As a rule, therefore, free carboxyl groups in proteins must be derived from dicarboxylic acids. Now gelatin, according to the analyses of Dakin [21] contains 0.907 per cent of nitrogen in the form of the dicarboxylic acids, aspartic acid and glutamic acid. To neutralize the free carboxyls of these acids, assuming that in each case only one carboxyl binds the amino acid to the protein molecule, would require 64×10^{-5} equivalents of base per gram of gelatin. This allows nothing for internal neutralization of dicarboxylic acids by diamino acids which, leaving lysine out of consideration, furnish 16 per cent of the total nitrogen in gelatin, nor have we made any allowance for the neutralization of "amid" nitrogen in gelatin. Yet the actual neutralizing capacity of gelatin for bases, as Loeb has shown [81] equals or exceeds 100×10^{-5} equivalents of base per gram (at pH 11.5).

Uncombined casein is insoluble in water, but when combined with acids or with bases it is soluble. When just sufficient alkali has been employed to carry every particle of casein into solution then at least one molecule of the alkali must have combined with each molecule of casein. Now Robertson [105] and also Van Slyke and Bosworth [132] have shown that to carry one gram of casein into solution 11.4×10^{-5} equivalents of base, or 1.14 cc. of tenth normal alkali just suffice, indicating a combining weight for casein

of about 8800. The tyrosine and cystine contents of casein indicate that its molecular weight must be some multiple of 4400.

In the presence of *excess* of alkali, however, the combining capacity of casein for bases is very much greater. This we may ascertain by titration of casein solutions, employing indicators which change color at pH values in excess of that at which casein is just held in solution (approximately 5). Thus at neutrality to litmus (pH = 7.2) casein neutralizes 50×10^{-5} equivalents of alkali per gram, while at neutrality to phenolphthalein (pH = 8.2) it neutralizes 80×10^{-5} equivalents of alkali per gram [105]. The employment of indicators for this purpose limits us, however, to the particular pH values at which the chosen indicators change colour. Employing the hydrogen electrode, by which means we can obtain a continuous curve of neutralization, we find that in the presence of excess of alkali the combining capacity of casein for bases approaches a constant maximum of 180×10^{-5} equivalents of base per gram [105]. We have seen that the minimal combining capacity of casein for bases is 11.4×10^{-5} equivalents of base per gram. Hence, reasoning as we did in the case of the compounds of edestin with chloric acid, if the minimal proportion of alkali which just suffices to carry casein into solution corresponds to the union of one molecule of alkali with one molecule of casein, the maximal proportion of alkali which may be bound by casein must correspond to the union of at least $\frac{180}{11.4} = 16$ molecules of base with one molecule of protein. If these molecules of alkali were united to the protein through carboxyl groups then there must be 32 oxygen atoms contained in free carboxyl groups in every equivalent of casein weighing 8800 grams, that is, 5.82 per cent of casein must consist of oxygen atoms in free carboxyl groups, in other words, 25 per cent of all the oxygen in casein would be present in this form. Now it must be recollected that there are no free amino groups in casein other than those which constitute the ω-amino groups in lysine. The α-amino groups in lysine are neutralized by carboxyl groups and so are the α-amino groups in all of the other amino acids which participate in the construction of the protein. Just as many carboxyl groups must have been neutralized as amino groups and therefore since each amino acid has contributed at least one amino group for neutralization, each amino acid must also have contributed one carboxyl group. Only those carboxyls can be free, therefore, which are contributed by the second carboxyl of the dicarboxylic acids, glutamic acid, hydroxy glutamic acid and aspartic acid. Now the analyses of Osborne and Guest and those of Dakin show that casein contains 15.6 per cent of glutamic acid, 10.5 per cent of hydroxyglutamic acid, and 1.4 per cent of aspartic acid [20, 92]. The oxygen contained in the second carboxyls of these amino acids would collectively amount to 5.8 per cent of the total weight of casein, a figure which at first sight appears to account very satisfactorily for the capacity of casein to combine with bases. Against this, however, we must set the ammonia which casein and other proteins yield on hydrolysis (the "amid" nitrogen) and which can only be combined with carboxyl groups which are not otherwise neutralized. Now the ammonia which casein yields on hydrolysis is 1.6 per cent of its weight [92] which would neutralize carboxyls containing 3 per cent of oxygen. Subtracting this from the above, we are left with a maximum of 2.8 per cent of oxygen contained in free carboxyl groups, while the neutralizing capacity of casein for

bases, if it were attributable to carboxyl groups, would require, as we have seen, the presence of 5.82 per cent of oxygen in this form.

One method of escape from this conclusion would be to suppose that proline and hydroxyproline, which contain no amino groups, always form terminal elements of the protein molecule, which would permit the presence of a corresponding number of free carboxyl groups at other extremities of the molecule. The content of proline in casein is stated by Osborne and Guest and by Van Slyke [92, 127] to be 6.7 per cent, and of hydroxyproline 0.3 per cent. One carboxyl group corresponding to every atom of nitrogen in this amount of proline and hydroxyproline would correspond to 1.94 per cent of oxygen which, added to the previous total, yields 4.74 per cent of carboxyl oxygen, which is still 1.06 per cent short of the required amount. As a matter of fact, however, this supposition cannot be entertained because it would involve the consequence that the capacity of protein to neutralize bases would be proportional to its content of proline and hydroxyproline. The capacity of gelatin to neutralize bases should therefore be greatly in excess of the base-neutralizing capacity of casein (since gelatin contains 9.5 per cent of proline and 14.1 per cent of hydroxyproline), whereas the reverse is actually the case. Similarly the phosphorus content of casein will not suffice to account for the neutralizing capacity of casein, even if we assume that the phosphoric acid is only united to the casein molecule by one of its hydroxyl groups, for 0.8 per cent of phosphorus [131] would in that case provide 0.83 per cent of oxygen in the form of free hydroxyl groups, which would be equivalent in neutralizing capacity to 1.65 per cent of oxygen in carboxyl groups. Added to the previously ascertained maximum residue of 2.8 per cent of oxygen contained in free carboxyl groups this yields 4.45 per cent which is still 1.37 per cent short of the required total. The estimate of the neutralizing capacity of casein cannot possibly be in error to anything approaching this extent. We have, moreover, made no allowance for internal neutralization of the diamino radicals in the protein and we have not hesitated to adopt any assumption, whether inherently probable or not, which would lend encouragement to the belief that free carboxyl groups accomplish the neutralization of bases by proteins, and we are still unable to account for the whole of the neutralizing capacity of casein for bases. In fact no reasonable estimate of the free carboxyl content of casein will account for more than one-half its actual neutralizing capacity.

It has been shown by Bosworth [11] that fibrin just dissolves when combined with 15×10^{-5} equivalents of alkali per gram. At neutrality to phenolphthalein ($pH = 8.2$) it neutralizes 61.5×10^{-5} equivalents of alkali per gram. What further capacity for neutralizing bases it may develop at higher pH values has not yet been ascertained, but the above figures show that some multiple of 4 and very probably more molecules of non-acid base must be neutralized at neutrality to phenolphthalein by one molecule of fibrin. If the first compound corresponds to the union of *one* molecule of base with one molecule of fibrin, then the oxygen in free carboxyl groups must constitute 1.92 per cent of the weight of fibrin. The amid nitrogen yielded in the hydrolysis of ox-fibrin, according to Gortner and Wuertz [38] is 9.3 per cent of the total nitrogen or 1.47 per cent of the weight of fibrin. This would correspond to a further 3.36 per cent of oxygen in the form of carboxyl groups, making a total of 5.28 per cent, corresponding to 24.25 per cent of glutamic acid. The actual glutamic acid content of fibrin is stated by Abder-

halden and Voitinovici [3] to be 10.4 per cent and by Levene and Van Slyke [74] 6.6 per cent. If we add the aspartic acid content ascertained by Abderhalden and Voitinovici (instead of the lower figure obtained by Levene and Van Slyke) and compute this in terms of glutamic acid, the total corresponds to 12.6 per cent of glutamic acid and still provides only half the free carboxyls which would be required. Unfortunately we have no estimate of the hydroxyglutamic acid content of this protein, and the supposition may therefore be entertained that the residual combining capacity which is still unaccounted for may be due to the second carboxyl group of this amino acid. But, on the other hand, the combining capacity of fibrin for bases, upon which the above comparison is founded, is certainly far short of its maximal combining capacity and, as in the case of casein, we have made no allowance whatever for possible internal neutralization of the "diamino" acids, arginine and histidine.

From all these results, and others of like nature which might be cited, we must certainly infer that the capacity of proteins to neutralize acids and bases cannot be wholly attributed to terminal amino or carboxyl groups. We are unable, it is true, to state that these play no part whatever in the neutralization of acids and bases, on the contrary many proteins demonstrably possess free amino groups and these must doubtless exercise their basic function in the presence of a sufficient excess of acid. We lack information concerning the existence or otherwise of free carboxyl groups in the protein molecule, but if they are present they, too, must doubtless contribute their share towards the total neutralizing capacity of the protein in solutions which contain a sufficient excess of free base. But in each case the number of these groups is demonstrably insufficient to account for the total neutralizing capacity of the proteins. Now we cannot hope to attain a full and true knowledge of the nature of the compounds which proteins form with inorganic acids and bases if we remain ignorant of the source of so considerable a proportion of their neutralizing capacity. We must now consider the question, therefore, to what elements of the protein molecule, other than terminal amino or carboxyl groups, we may attribute this residual combining capacity.

3. The Chemical Origin of Neutralization by Proteins

Since we cannot attribute the residual combining capacity of proteins for bases to carboxyl groups its origin at first sight seems difficult to perceive, but the investigations of Osborne and Leavenworth [94] enable us to assign its origin to COHN groups in the interior of the protein molecule.

It has been shown by Kober [54, 55, 56, 57] that the copper salts of amino acids in alkaline solution yield their copper quantitatively in the form of a precipitate of cupric hydrate on heating the solution or on addition of an excess of alkali. Under similar conditions the copper compounds of peptones and peptides yield little or no precipitate. Osborne and Leavenworth have shown that the maximum amount of cupric hydrate which edestin or gliadin will hold in solution *exactly corresponds with the number of COHN groups in the molecules of these proteins,* assuming that one atom of copper combines with each atom of nitrogen. In the case of the union of cupric hydroxide with proteins, therefore, the combination is effected by means of the COHN groups within the body of the protein molecule, and the supposition may therefore be entertained that the combination of proteins with other bases is effected in a like manner.

That the neutralization of acids may similarly be effected by — COHN-groups is shown by the observations of Siegfried upon the barium salts of carbamino acids which are formed when carbon dioxide is passed into solution of amino acids in dilute barium hydroxide [120]. Direct analysis of the products obtained when $BaCO_3$ or $CaCO_3$ act upon glycocoll and other amino-acids showed that these compounds can be represented by the general formula:

$$
\begin{array}{c}
\text{H} \\
/ \\
\text{N} \\
/ \quad \backslash \\
\text{R} \quad \text{COO} \\
| \qquad | \\
\text{COO——Ba}
\end{array}
$$
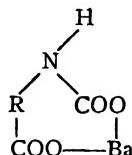
being the barium or calcium salts of carbamino acids. On standing barium carbonate or calcium carbonate, as the case may be, is slowly liberated, and this process is accelerated by heating.

For monoamino acids the ratio:

$$\frac{\text{Molecules } CO_2 \text{ bound}}{\text{atoms of N}}$$

is 1, indicating that the — NH_2 group reacts quantitatively with the carbonate. For diamino acids such as lysine, the ratio is also 1, showing that both — NH_2 groups react quantitatively. For arginine, which contains four atoms of nitrogen and to which the ordinarily accepted formula ascribes two amino groups, the ratio is $\frac{1}{4}$, indicating either that only one of the amino groups reacts, and neither of the amino groups, or, which is more probable, that the true structure of arginine contains only one amino group, the guanidine nucleus having a structure analogous to that of urea [138].

For the different dipeptids Siegfried found that the ratio varies between

$$\frac{1}{1.63} \quad \text{and} \quad \frac{1}{1.79}$$

If the — NHOC-groups did not react at all, the ratio would be $\frac{1}{2}$, if they reacted quantitatively it would be 1. For tripeptides the ratio is $\frac{1}{2.57}$, whereas it would be $\frac{1}{3}$ if the NHOC groups did not react at all. For the tetra-peptide, triglycyl glycine, the ratio is $\frac{1}{3.29}$, whereas it would be $\frac{1}{4}$ if the — NHOC-groups did not react. It is therefore clear that COHN groups in the various polypeptides are capable of combining with even so weak an acid as carbonic acid in aqueous solution. There is no reason to suppose that COHN groups in proteins behave differently from those in the peptides, in fact the above-quoted investigations of Osborne and Leavenworth show that in other respects the COHN groups in proteins act in precisely the same way as the COHN groups in peptides, and, of course, their capacity for binding strong acids in aqueous solution must be much greater than their capacity for binding such an extremely weak acid as carbonic acid.

It might seem at first sight much more reasonable and natural to suppose

that the residual combining capacity of proteins for acids is attributable to the imino group in histidine or the guanidyl radical in arginine. Consideration of the hydrolytic dissociation which these salts would undergo in aqueous solution, however, at once renders this supposition untenable. It has been shown by Kanitz [53] that whereas histidine monohydrochloride, formed by the union of hydrochloric acid with the α-amino group, is only 0.58 per cent hydrolytically dissociated in tenth molecular aqueous solutions, the dihydrochloride, formed by the union of hydrochloric acid with the imino group, is 32 per cent dissociated in tenth molecular and 75 per cent dissociated in one-hundredth molecular aqueous solutions. Now, taking the case of gelatin, which has a minimal molecular weight of the order of 10,000, a 1 per cent solution represents a one-thousandth molecular concentration, and if this weight of gelatin contains, as D. Jordon Lloyd has estimated [78] two histidine radicals, the concentration of histidyl groups in a 1 per cent solution of gelatin can only be $\frac{1}{500}$ molecular. It is obvious that at this dilution histidyl hydrochloride, formed by the union of hydrochloric acid with the imino group, would be almost completely dissociated into histidyl (united by the α-amino group with the protein) and free hydrochloric acid.

In the case of arginine, since it contributes no free amino groups to the protein molecule, it might be supposed that it is united to two carboxyl groups. As a matter of fact, however, free arginine itself yields nitrogen, on treatment with nitrous acid, corresponding to only one amino group [126]. We may therefore be confident, as E. A. Werner has indicated [138] that only one amino group is actually present in arginine, namely the α-amino group which binds it to the protein molecule. The researches of Werner, besides necessitating a reconstruction of our conception of the structural formula of urea, have also rendered untenable our former ideas of the structure of other guanidine derivatives including, of course, arginine itself. If the formula of arginine were analogous to that of urea we would have to write it:

$$HN = C <\begin{matrix} NH_2 \\ | \\ N.CH_2.CH_2.CH_2.CH(NH_2)COOH \end{matrix}$$

If this be the true structure of arginine then the point of attachment of the second nitric acid molecule in arginine dinitrate must be the imino group. In any case, whatever the mode of attachment, the hydrolysis which this compound undergoes in aqueous solution would render it incapable of existence to any important extent in the solutions of protein which are commonly employed for determining their capacity for neutralizing acids. Thus Kanitz finds that whereas arginine mononitrate does not undergo measurable hydrolytic dissociation even in one-thousandth molecular aqueous solution, the dinitrate is 19.5 per cent dissociated even in tenth molecular solution, so that in $\frac{1}{450}$ molecular solution it would be 75 per cent dissociated. The arginine in casein (3.8 per cent) corresponds to 0.00218 mol. of arginine in every litre of one per cent solution of casein. In this solution, therefore, the arginyl radicals would be present in $\frac{1}{459}$ molecular concentration and the hydrochloride would be 75 per cent decomposed leaving a residue of 0.00054 equivalents per liter or 5.4×10^{-6} equivalents of acid bound by one gram of

casein at a pH of 2.8. This is only about one-eighth of the acid which is actually bound by casein at pH = 3 [49].

We have hitherto assumed, as though it were self-evident, that the amino groups which are actually present in proteins contribute an important quota towards their neutralizing capacity for acids, but, in fact, this is very far from being the case. We have seen that the free amino groups in proteins are the ω-amino groups of the lysine radicals of which the α-amino groups form the means of junction with the protein. The difficulty with which the ω-amino group in lysine is oxidized by nitrous acid indicates that the nitrogen atom in this group possesses only a feeble affinity for positive charges [34], that is, is but feebly negative, and we would therefore expect this group to display only very weak basic properties. The measurements of Kanitz confirm this anticipation, for while lysine monochloride, formed by the union of hydrochloric acid with the α-amino group, does not undergo any measurable degree of hydrolytic dissociation, even at a dilution of one-thousandth molecular, the dihydrochloride is decomposed to the extent of 22 per cent in tenth normal solution and 86 per cent in one-thousandth normal solution. The percentage of amino nitrogen in casein, as ascertained by Van Slyke and Birchard, corresponds to the presence of 0.006 equivalents of lysyl groups in a liter of 1 per cent casein solution. The ω-amino group of lysine is therefore present in such a solution in $\frac{1}{167}$th molecular concentration, from which it follows that the hydrochloric acid compound formed with this group must be 64 per cent dissociated, leaving a residue of 21.6×10^{-5} equivalents of acid actually bound by these amino groups at a pH of 2.4. This is only two-fifths of the amount of acid bound by casein at this pH according to the determinations of Loeb [81] and only one-fifth of the maximum capacity of casein for binding acids.

The possibility will naturally occur to the reader that the basic properties of the ω-amino group in lysyl are enhanced by the union of the α-amino group with protein, so that its salts no longer undergo the same degree of hydrolytic dissociation in aqueous solution as the salts of the ω-amino group in free lysine. It has been pointed out by Fry, however, that the susceptibility of the amino group to oxidation by nitrous acid is a measure of its affinity for acids [34, pp. 69 to 72]. If the ω-amino group in the lysyl groups of proteins had undergone an increase of basicity ($=$ negativity), therefore, it would also have become more susceptible to oxidation by nitrous acid. Now it has been shown by Van Slyke [126] that the time required for the complete interaction of the free amino groups in protein with nitrous acid is much longer (30 minutes) than that required for the complete interaction of α-amino groups, but that it is equal to that required for the complete oxidation of the ω-amino group of lysine. Therefore we may conclude that the ω-amino group in the lysyl radicals in proteins has not undergone any appreciable accession of basic properties.

The results of these and similar calculations are summarized in the accompanying table. The molecular concentrations of the arginyl and histidyl groups in 1 per cent solutions of the proteins are calculated from the determinations of Dakin [21] and Van Slyke [128, 129]. The molecular concentrations of the lysyl groups are computed from the determinations of Van Slyke and Birchard. It will be seen that the united combining capacity of these groups does not exceed one-third of the total combining capacity of

Protein	Molecular Concentration × 10³ of Amino-acid Radicals in 1 per cent Solution			Equivalents of Acid × 10⁵ Bound by One Gram of Protein Due to:			Total × 10⁵	Actual Combining Capacity × 10⁵	Residual Combining Capacity × 10⁵
	Lysyl	Arginyl	Histidyl	Lysyl	Arginyl	Histidyl			
Gelatin	4.0	4.71	0.58	11.6	17.9	0.2	29.7	92.5	62.3
Casein	6.0	2.18	1.61	21.6	5.4	1.4	28.4	100	71.6
Edestin	2.6	8.28	1.55	6.0	38.1	1.4	44.5	180	135.5

gelatin or casein for acids, or one-fourth of the combining capacity of edestin. Moreover, none of these compounds could exist to any appreciable extent at pH values below 3. Practically the whole of the combining capacity of edestin determined by T. B. Osborne at neutrality to Tropaeolin (pH = 3) [87] must therefore be attributable to groups other than arginyl, histidyl or the ω-amino group of lysyl.

What we have termed the "residual" combining capacity of proteins for acids and bases is therefore that part of their combining capacity which is exhibited in solutions of which the reaction does not depart far from neutrality, as when casein combines with bases to form acid solutions of the caseinates [105], or when edestin combines with 180×10^{-5} equivalents of acid per gram at a pH of 3.

Since the ω-amino group in lysine has very weak basic properties, its salts are liable, as we have seen, to extensive hydrolytic dissociation in aqueous solutions. On the other hand the α-amino groups of amino-acids are strongly basic and their salts are comparatively little subject to hydrolytic dissociation. Hence, if the neutralization of acids by proteins were actually attributable to the terminal amino groups which they contain, their capacity to neutralize acids should be greatly enhanced by hydrolysis, and the ratio:

$$\frac{\text{Equivalents of acid bound}}{\text{Equivalents of free amino nitrogen}}$$

should *rise* in consequence of hydrolysis. Similarly we would expect, after the analogy of other dibasic organic acids, to find the salts formed by combination of bases with the free carboxyls of dicarboxylic acid radicals in proteins subject to a considerable measure of hydrolytic dissociation, while those formed by the monocarboxylic acids set free in hydrolysis should be much less subject to hydrolytic dissociation. Since the carboxyls uncovered by hydrolysis must be equivalent to (or slightly in excess of) [4] the amino groups set free, the ratio:

$$\frac{\text{Equivalents of alkali bound}}{\text{Equivalents of free amino nitrogen}}$$

should also *increase* very greatly after hydrolysis of the protein.

If, on the other hand, the neutralization of acids and bases is accomplished by internal COHN groups in the protein molecule, then the opening of these groups by hydrolysis might result, either in no change of the ratios:

$$\frac{\text{Equivalents of acid or alkali bound}}{\text{Equivalents of free amino nitrogen}}$$

[4] On account of the presence of proline and oxyproline radicals which are united to the carboxyls of adjacent acids, not by amino, but by imino groups, which do not react with nitrous acid.

or in a *decrease* of these ratios, if the compounds formed with free amino or carboxyl groups in the products of hydrolysis chance to be more subject to hydrolytic dissociation than the compounds formed with the unhydrolysed protein. The absolute capacity for neutralizing acids and bases might usually be expected to increase, because not all of the COHN groups in protein participate simultaneously in the neutralization of acids and bases [5] and some of these, not effective for this purpose in the unhydrolysed protein, might be opened up by hydrolysis and become effective.

The change in the ratios:

<div align="center">
Equivalents of acid or alkali bound
Equivalents of free amino nitrogen
</div>

consequent upon hydrolysis, should therefore indicate the predominant method of combination of proteins with acids and bases. Unaltered or decreased ratios would indicate that the unhydrolysed protein binds acids and bases through internal COHN groups, while increased ratios would suggest that unhydrolysed protein binds acids and bases through terminal amino and carboxyl groups.

The author has investigated the effect of hydrolysis upon gelatin and casein from this point of view [112]. Hydrolysis was achieved through the agency of trypsin of which the neutralizing-capacity and amino-nitrogen content were separately determined and subtracted from the results. The proteins were dissolved in one per cent concentration and the percentage of the total nitrogen which was present in free amino groups was determined, before and after hydrolysis, by formol-titration. Hydrolysis was only partial, but the amino nitrogen content had increased, in the case of gelatin, from 2.50 per cent of the total nitrogen to 9.05 per cent, and, in the case of casein, from 3.63 per cent to 14.64 per cent. Three pH values were employed for the comparison, namely, pH = 2; pH = 8.2; and pH = 10.5. These correspond respectively, to the red tint of thymol blue, faint pink of phenolphthalein, and faint but decided blue of thymolphthalein. Solutions having these pH values were made up and a given proportion ($\frac{1}{10}$ cc. of 0.4 per cent solution per 10 cc.) of the indicators added. The same proportion of indicator was added to the protein solutions and acid or alkali added from a burette until the color matched the standard. The uncombined acid or alkali necessary to produce the observed pH was subtracted from the total amount of acid or alkali added, the residue representing the acid or alkali bound by the protein. The following were the results obtained:

pH	Ratio of Equivalents of Acid or Base Neutralized to Equivalents of Free Amino Nitrogen:			
	Gelatin		Casein	
	Before Hydrolysis	After Hydrolysis	Before Hydrolysis	After Hydrolysis
2.0	2.77	1.98	4.00	0.40
8.2	1.25	0.72	1.97	0.91
10.5	1.65	1.99	2.53	1.99

[5] Otherwise the neutralizing capacity of proteins for acids and bases would be much greater than it actually is.

It will be seen that in every instance other than gelatin at the highest pH (10.5) the effect of hydrolysis is to *reduce* the ratio of the combining capacity to the equivalents of amino nitrogen. We must infer, therefore, that with the possible exception of gelatin at pH = 10.5 the predominant method of combination of these proteins with acids and bases is not through free amino or carboxyl groups, but through internal COHN groupings. The occurrence of ratios in excess of unity also indicates that neutralization is achieved by agencies other than free amino or carboxyl groups.[6]

It is a remarkable fact that the combining capacity of casein for acids at pH = 2 fell from 16.3 cc. of tenth normal hydrochloric acid per gram of protein to 6.6 cc. of tenth normal hydrochloric acid per gram in consequence of hydrolysis. Since the free amino groups had simultaneously increased nearly five hundred per cent this fact demonstrates most clearly that free amino groups do not accomplish the neutralization of acids by casein. On the other hand, those groups which are *diminished* by hydrolysis are COHN groups.

Shortly after the author first advanced the view that a considerable proportion of the combining capacity of proteins for acids is not attributable to free amino groups [110], Blasel and Matula investigated the acid-binding capacity of deaminized gelatin [9]. Their results indicated that gelatin which has been deaminized by treatment with nitrous acid retains to the full the acid-binding capacity of untreated gelatin, a result which was somewhat surprising, since it could hardly have been expected that the terminal amino groups which are known to be present in gelatin would play no part whatever in determining its maximal combining capacity. Hitchcock, however, employing an improved method of deaminization, finds that the maximal combining capacity of gelatin for acids is reduced to one-half by deaminization and that the loss of combining capacity is exactly equivalent to the amino groups which have been destroyed [49], a result which, again, is somewhat surprising because one would have expected the loss of combining capacity to be considerably less than equivalent to the loss of amino groups, on account of the hydrolytic dissociation which the compounds of these amino groups with acids·might be expected to undergo, even in solutions which contain an excess of acid. Thus, according to Hitchcock, the maximum combining capacity of gelatin is attained at pH = 1.8, whereas lysine dichloride is 30 per cent dissociated at this pH even in twentieth molecular concentration and must be considerably more dissociated in $\frac{1}{250}$th molecular concentration (1 per cent gelatin) even if the pH is maintained unaltered. It is impossible at present, however, to ensure that no changes other than deamination are brought about in proteins by nitrous acid in acid solution and quantitative comparisons of this kind are therefore subject to this measure of uncertainty.

Accepting Hitchcock's figures, however, it is clear that a large proportion of the combining capacity of gelatin for acids and a still larger proportion of the combining capacities of casein and edestin for acids cannot be accounted for by the terminal amino groups contained in these proteins, even if we add to these the arginyl and histidyl radicals which they also contain.

---

* Since hydrolysis was incomplete, that is, the proteins were not completely converted into amino acids, there were still COHN linkages, although fewer of them, present in the hydrolysed mixtures. Hence those ratios which were most greatly in excess of unity in the unhydrolysed protein, approached unity but did not always attain it after hydrolysis.

We have seen that the combining capacity of proteins for bases is similarly unaccountable in terms of the free carboxyl groups which they may possibly contain. The results of Osborne and Leavenworth and those of Siegfried, cited above, indicate that the COHN groups in the interior of the protein molecule are capable of uniting both with bases (cupric hydroxide) and with acids (carbonic acid), the latter result indicating, when we consider the weakness of carbonic acid, that the resultant compounds are not subject to hydrolytic dissociation.[7] We are thus led to ascribe the residual combining capacities of proteins to the COHN groups which they contain. We must now consider, therefore, the mechanism whereby these groups may accomplish the neutralization of acids and bases. In order to discuss this question adequately, however, we must first undertake a review of certain facts which necessitate a revision of the current conception of the structure of the protein molecule.

4. THE CYCLIC DIKETOPIPERAZINE STRUCTURE OF THE CONSTITUENT UNITS OF THE PROTEIN MOLECULE

While the researches of Emil Fischer have rendered it quite certain that the α-amino groups of the constituent amino-acid radicals in protein are united to the carboxyl groups of other, similar radicals, the arrangement of these radicals in the molecule is still unknown even in the synthetic polypeptides. It is commonly assumed, following the suggestion of Hofmeister [50] that the amino-acids are linked together in long chains with few or no branches, having the following type of structure:

$$
\begin{array}{l}
\text{C} \dots \dots \dots \text{C} - \text{CO} \\
\qquad\qquad\qquad\quad \text{NH} \\
\text{C} \dots \dots \dots \text{C} - \text{CO} \\
\qquad\qquad\qquad\quad \text{NH} \\
\text{C} . \; \dots \dots \dots \text{C} - \text{CO} \\
\qquad\qquad\qquad\quad \text{NH} \\
\text{C} \dots \dots \dots \text{C} - \text{CO} \\
\qquad\qquad\qquad\quad \text{NH}
\end{array}
$$
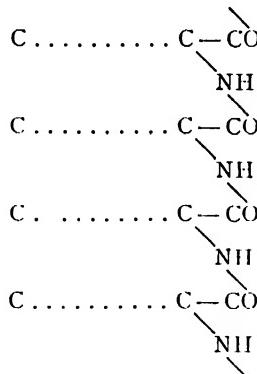
If we carefully consider the consequences which must follow from this type of structure, however, two facts become apparent. The first is that the structure should be very unstable owing to the magnitude of the strains which must develop in an open chain of such great length, and the second that, no matter what its length, at its terminations must appear a free amino and carboxyl group respectively. We can readily understand how, in a structure of this type, the ω-amino groups of lysyl radicals may remain uncombined, but we fail to understand why, in addition to these, there does not appear

[7] The compound of gelatin with hydrochloric acid is not subject to hydrolytic dissociation since, as Hitchcock has shown, the combining capacity of gelatin for hydrochloric acid is independent of its dilution [49]. The author had previously shown that the caseinates of the alkalies are similarly not subject to hydrolytic dissociation [105], *see also* Pauli and Hirschfeld [96].

at least one other free amino group, and that an α-amino group, situated at the extremity of the molecule.

It might be suggested that the free amino group at one extremity of the molecule unites with the free carboxyl at the other, to form a closed ring. But all our experience indicates that a closed ring of this magnitude could not exist. The internal strains to which it would be subject would disrupt it. Rings containing great numbers of carbon and nitrogen atoms do not occur and, in fact, as von Baeyer originally pointed out, the most stable ring-formations are those which contain five or six carbon or carbon and nitrogen atoms. The latter type of ring would correspond to the structure of the diketopiperazines.
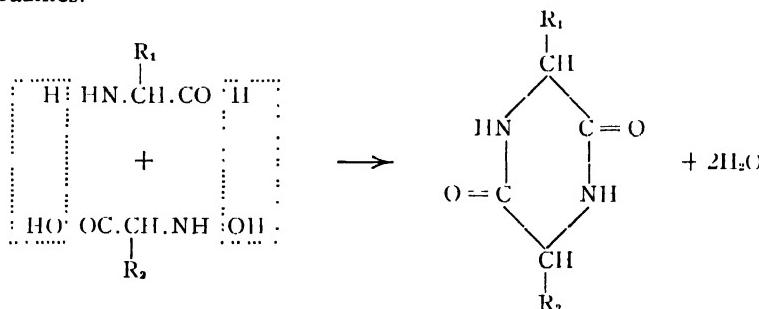
The probability that diketopiperazine rings form an important proportion of the protein molecule was frequently emphasized by Emil Fischer, and this supposition has received unexpected verification from the recent investigations of H. R. Marston, conducted in this laboratory [83].

It was discovered by the author in 1907 that if safranine be added to a neutral or faintly alkaline solution of trypsin "a light, flocculent colored precipitate appears on standing and gradually settles" [101]. The action of safranine solutions upon trypsin was subsequently further investigated by Holzberg [51] who showed that the precipitate, which is only very sparingly soluble in water, contains the whole of the proteolytic activity of the trypsin solution and that the residue of inactive material which is not precipitated by safranine forms a very large proportion of even the purest preparations of pancreatin. Holzberg was unable to separate the trypsin from its combination with safranine without destruction of its proteolytic activity. J. T. Wood subsequently confirmed the observations of Holzberg but was unable to obtain any precipitate from solutions of highly purified trypsin [141]. This was due, however, to his failure to employ centrifugalization. The precipitate, especially in dilute and highly purified solutions of trypsin, is extremely light and forms flocculi which only very slowly settle and are partially transparent, so that in small amount the precipitate may not communicate any appreciable opacity to the fluid. Small amounts of precipitate readily pass through filter paper, but energetic and prolonged centrifugalization will remove it quantitatively from the fluid. Phosphates and nucleic acid also yield precipitates with safranine but they are more soluble than the enzyme compound and, moreover, the precipitate formed in solutions which are contaminated by neither of these impurities.

Safranine is diamino-phenyl-tolazonium chloride [119].
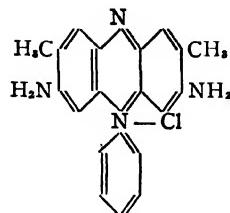
This fact led Marston to investigate the action of other azine and azonium dyes (eurodines, safranines and indulins) upon solutions of trypsin. Among the series of dyes investigated all those which were soluble in water and contained an azine nucleus were found capable of completely precipitating the enzyme from its solution. Thus neutral red (dimethyl-diamino-toluphenazine hydrochloride) :
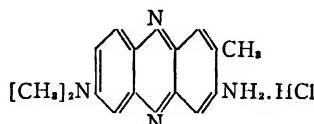
neutral violet (dimethyl-diamino-phenazine hydrochloride) :
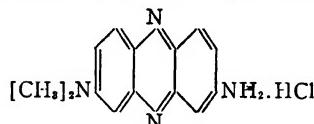
pheno safranine (diamino-phenylphenazonium hydrochloride) ; Safranine MN (dimethyl diamino-phenyl-phenazonium hydrochloride) :
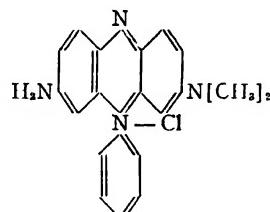
and indamine blue (amino-dianilido-phenyl-phenazonium chloride) :
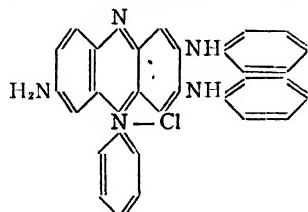
all brought about removal of the enzyme from the solution.

From this Marston infers that the enzyme enters into combination with the nitrogens of the azine nucleus, in confirmation of which he adduces the fact that the combination of safranine with trypsin involves a tautomeric

change of internal linkages which is evidenced by change of color. The precipitate is, in fact, not red but violet. Moreover, the reduced leuko-safranine:

Dye chloride (red).   Leuko base (colorless)
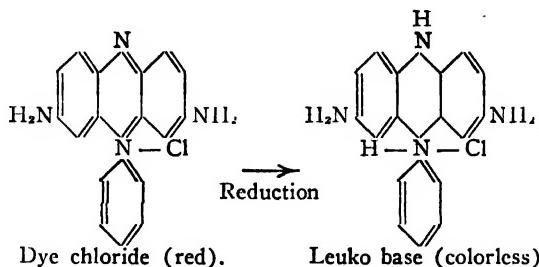
in which hydrogen atoms have taken up the positions which would presumably be occupied by the enzyme in the compound, has completely lost the ability to precipitate trypsin.

Not only trypsin, however, but all of the proteolytic enzymes are precipitated from their solutions by the water-soluble azine and azonium dyes. Thus pepsin, erepsin, papain and the endo-tryptase of yeast are removed from solution by the addition of pheno-safranine and the resultant precipitate contains the whole of the proteolytic activity of the enzyme. On the other hand, the azine precipitates from crude pancreas extracts, while strongly active proteolytically, display no lipolytic or diastatic activity, although both of the enzymes responsible for these activities could be demonstrated with ease in the original extracts. These precipitating agents are therefore specific for the proteolytic enzymes.

But if the proteolytic enzymes, and no other hydrolyzing enzymes, enter into firm combination with the nitrogens in azine nuclei, then, since we know that proteolytic enzymes also enter into combination with the proteins prior to the hydrolytic splitting which they induce [23, 24, 33, 48, 86, 135], the probability is very strongly indicated that the proteins themselves contain azine nuclei which form those portions of the protein molecule which are susceptible to attack by the proteolytic enzymes. The frequent presence of diketopiperazines among the products of the partial hydrolysis of proteins [1, 2, 32, 42, 73, 116] the absence of free amino groups in proteins other than the ω-amino groups of lysine and the susceptibility of proteins to hydrolysis by the proteolytic enzymes, all unite in pointing, therefore, to the existence of an important proportion of diketopiperazine rings in the protein molecule.

If the amino acids in the protein molecule are united with one another in diketopiperazine rings this must obviously lead to the neutralization of all of the amino groups in the constituent radicals of the protein molecule excepting those which are contained in diamino (lysyl) radicals. The length of the amino acid chain would be greatly reduced in comparison with the open-chain formula and the strains sustained by the bonds uniting the various elements of the chain would be proportionally diminished. The constituent diketopiperazine elements of the chain would themselves be possessed of an extremely stable configuration. Each of these rings would afford a possible means of attaching proteolytic enzymes and thus rendering the whole structure susceptible to hydrolysis by these enzymes.

The diketopiperazines, like the open-chain peptids, are capable of existing in either of two forms:

Keto Form   Enol Form
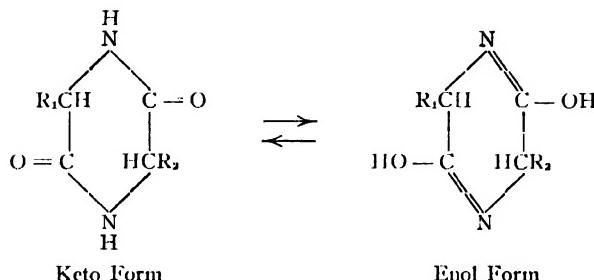
of which the enol form has the capacity of neutralizing bases through substitution of the hydrogen in the hydroxyl group by a metal or other basic radical. If two such rings unite the combined rings cannot, according to the octet theory of valency [68, 69, 71] contain more than 32 co-valency bonds, excluding those contained within the univalent radicals, R_1, R_2, etc. Any bonds in excess of this number, therefore, cannot be covalency bonds but bonds of electrostatic attraction, subject to modification by alterations of the dielectric constant of the medium in which the substance is dissolved, and to neutralization by the charges borne by other ions which the medium may chance to contain.

Bearing these facts in mind it is evident that there are only three ways in which stable union between pairs of diketopiperazine rings can be achieved. Two of these involve the union of pairs of diketopiperazines of the keto-form and the resultant compounds each contain 34 bonds of which two in each case must therefore be bonds of electrostatic attraction (indicated by dotted lines in the subjoined formulas). The third is accomplished by the union of two diketopiperazine rings of the enol-type and the resultant compound contains 32 bonds all of which are therefore true covalency bonds. The three possible methods of combination are represented by the following formulas:

First method of combination:
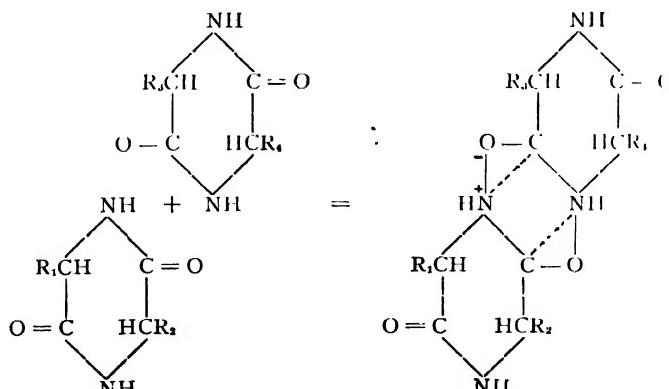
Second method of combination:
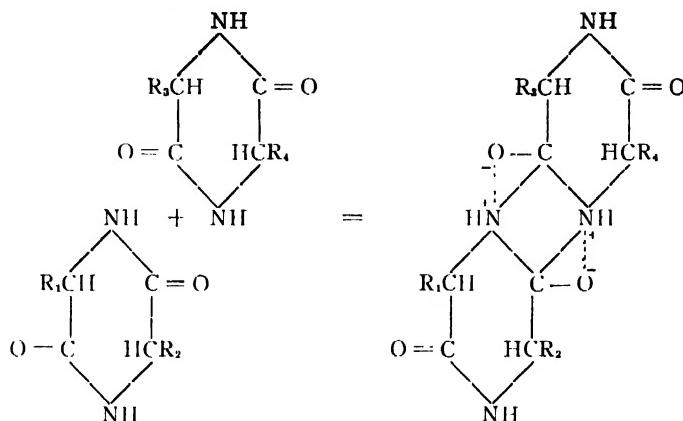
Third method of combination:
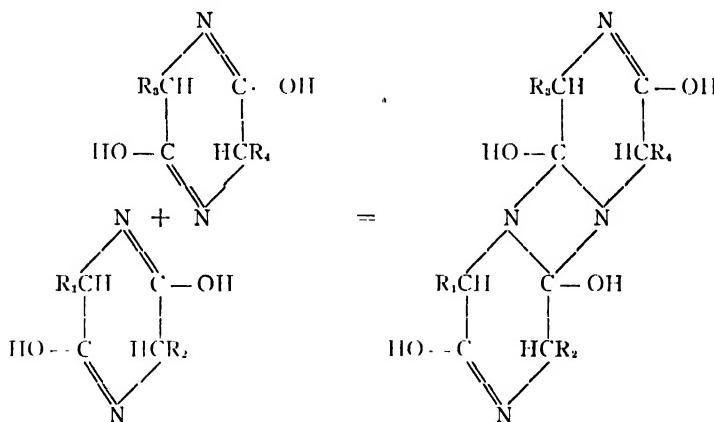
Of these three methods of combination only one, namely the second, would yield a compound displaying the properties of neutral (isoelectric) protein. If the other forms exist in acid or alkaline solutions, therefore, they must exist therein in tautomeric equilibrium with the product of the second type of combination. The following considerations will make this clear:

The compound of the first type affords no point of attachment for either acids or alkalis excepting the free COHN groups at the extremities of the molecule. Now it is evident that a chain of diketopiperazine rings could not be branched except in the event of a dicarboxylic acid radical uniting directly with a diamino radical which, as comparison of the dicarboxylic acid and diamino acid content of the proteins reveals, must be a comparatively rare event (because one of these types of radical usually predominates considerably over the other). A single—COHN—group at either extremity of the protein molecule would not, however, suffice to account for its residual combining capacity for acids and bases. Moreover, a compound of the first type

could not be split into its constituent open-chain dipeptids by the addition of hydrogen and hydroxyl ions derived from water, without previous dissolution of the bond between nitrogen and oxygen and consequent acquirement of the structure which would be attained by the second method of combination.

The third method of combination would yield a substance capable of uniting with bases by the substitution of a metal or a positive radical for the hydrogen in the hydroxyls. The author was formerly of the opinion that the enol type of structure of COHN groups would also suffice to account for the union of proteins with acids through the assumption of pentavalency (tetracovalency) by the nitrogen [111]. As a matter of fact, however, those peptides which are known to possess the enol type of structure are predominantly acid in character and Siegfried has shown that they are unable to combine with carbonic acid [120], and, presumably, therefore, they are unable to combine with other acids. It is more probable, as Jordon Lloyd has suggested [78], that the enol form of COHN groups in proteins exists only in alkaline solutions and is in tautomeric equilibrium with the keto-form which is assumed in acid solutions. This also enables us to comprehend the racemization of the amino acid radicals in proteins which, as Dakin has shown [19, 22, 25, 26, 142], occurs on prolonged standing in alkaline solutions, for if the hydrogen in the—COHN—group is permanently attached to the oxygen it is not clear why this hydrogen may gradually be replaced by hydrogen derived from the adjacent RHC group. But if the hydrogen is mobile, and the enol form in tautomeric equilibrium with the keto-form, then it is evident that its place may occasionally be taken by hydrogen derived from the RHC group. If this compound were more stable than the usual form, then the whole of the elements of the molecule which are susceptible to this change would slowly assume the more stable configuration, the carbon atoms in the corresponding RHC groups would lose their optical activity owing to their attachment to the adjacent carbons by double bonds, and the amino acids ultimately derivable from these diketopiperazine rings by hydrolysis would consist of optically inactive mixtures of the d and l-acids. This obviously corresponds with the phenomena observed by Dakin.

The compound yielded by the third method of combination would also not split into constituent dipeptides on hydrolysis with water unless the hydrogen first migrated from the O to the N in the—COHN—group, thus assuming the structure which would in any case be attained by the second method of combination. For if hydroxyl were to attach itself to the carbon without this preliminary change a dihydroxy compound would result and not a carboxyl group.

We are thus enabled to decide definitely that where diketopiperazine rings occur in the protein molecule they must be united to one another by the second of the methods outlined above, although in alkaline solutions the greater proportion of the linkages may actually assume the configuration depicted in the compound arising from the third method of combination.

The structure of the union between diketopiperazine rings which is thus indicated is one of remarkable interest. It is precisely analogous to the structure of urea [138] and, like urea, the compound thus formed must be chemically and electrically neutral. Now it has been shown by Pauli [95] that electrolyte-free serum albumin, that is, serum albumin which is uncombined with acids or bases, *is also electrically neutral*, although, addition of a trace of

acid to the solution causes the protein to wander towards the cathode under an electrical potential gradient, while a trace of alkali causes it to wander towards the anode. The converse of this experiment has been performed by Loeb [80], who has shown that isoelectric gelatin is also electrolyte-free.* On the other hand this mode of union between diketopiperazine rings involves the presence at each linkage of residual valencies which, in the isoelectric condition, are neutralized internally. In the presence of ionized substances in the solvent, however, the orientation of a proportion of these residual valencies will be directed externally towards the ions in the surrounding medium and hence, to the extent that the solvent medium is ionized, the protein must exhibit acid or basic qualities. Thus in the presence of the ions of water the following change of electronic orientation would occur:

$$
\begin{array}{c}
\diagdown\diagup \\
NH \\
\diagdown\diagup\ |^+ \quad + H^+ \ldots\ldots ^-OH \quad \rightleftarrows \\
C-O^- \\
|
\end{array}
\qquad
\begin{array}{c}
\diagdown\diagup \\
NH^+ \ldots\ldots ^-OH \\
\diagdown\diagup \\
C-O^- \ldots\ldots ^+H \\
|
\end{array}
$$
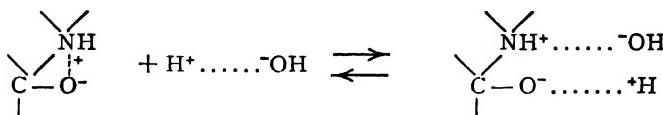
The extent to which this change would occur in pure water would be small and its measure would depend upon the distance to which the isoelectric point of the particular protein is removed from the pH at neutrality. But in the presence of excess of H$^+$ ions, for example, the extent of change would be much greater and, when sufficiently great, would lead to chemical combination in the following manner:

$$
\begin{array}{c}
\diagdown\diagup \\
NH^+ \ldots\ldots ^-Cl \\
\diagdown\diagup \\
C-O^- \ldots\ldots\ ^+H \\
|
\end{array}
\qquad \rightleftarrows \qquad
\begin{array}{c}
\diagdown\diagup \\
NHCl \\
\diagdown\diagup \\
C-OH \\
|
\end{array}
$$
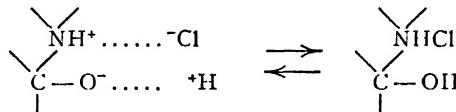
or, in the presence of excess of hydroxyl ions, to combination with bases as follows:

$$
\begin{array}{c}
\diagdown\diagup \\
NH^+ \ldots\ldots ^-OH \\
\diagdown\diagup \\
C-O^- \ldots\ldots ^+Na \\
|
\end{array}
\qquad \rightleftarrows \qquad
\begin{array}{c}
\diagdown\diagup \\
N \\
\diagdown\diagup \\
C-ONa \\
|
\end{array}
\quad + H_2O
$$
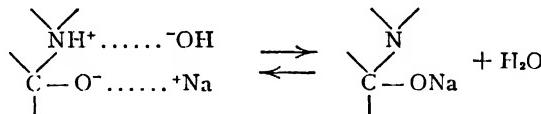
So that if "adsorption" be defined in the way that Langmuir has defined it [64, 66, 67], as attraction due to residual valencies, then we are in agreement with Bayliss [6] that adsorption is a preliminary step in the formation of chemical compounds with proteins.

5. THE IONIZATION OF PROTEIN SALTS.

Without attempting, within the limits of this chapter, any detailed consideration of the electrochemical behavior of protein salts, which has been discussed at length elsewhere [111], it may be pointed out that the method of combination of acids and bases with proteins, which has been depicted above, permits ionization of the resultant compounds in two different ways. The union of HCl or of NaOH with the complex represented by the formula:

* This is not *necessarily* the case. *J. A.*

$$NH$$

$$R_3CH \quad C = O$$

$$O - C \quad HCR_4$$

$$HN \quad NH$$

$$R_1CH \quad C -- O$$

$$O - C \quad HCR_2$$

$$NH$$
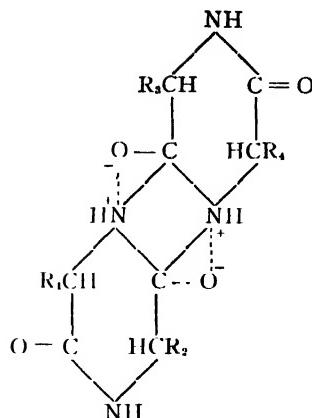
must result, in accordance with the octet theory of valency [68, 69, 71], in the increase of the total number of covalency bonds from 32 to 33. Hence, if the diketopiperazine rings remain united with one another, then in the chloride of the protein only the hydrogen can be held by a covalency bond, while the chlorine must be held by electrostatic attraction; in other words, the chloride of the protein must in that case, as in ammonium chloride itself [69] be *completely dissociated into chlorine ions and protein ions.* On the other hand, if both hydrogen and chloride are held by covalency bonds then one of the valencies uniting the diketopiperazine rings must become electrostatic and the compound will ionize *yielding two protein ions* in the following manner:

$$NH$$

$$R_3CH \quad C =- O$$

$$O - C \quad HCR_4$$

$$HN \quad NH$$

$$R_1CH \quad C -- O$$

$$O - C \quad HCR_2$$

$$NH$$

$$+ HCl =$$

$$NH$$

$$R_3CH \quad C = O$$

$$O -- C \quad HCR_4$$

$$- NHCl$$

$$HN$$

$$R_1CH \quad C^+ - OH$$

$$O = C \quad HCR_2$$

$$NH$$
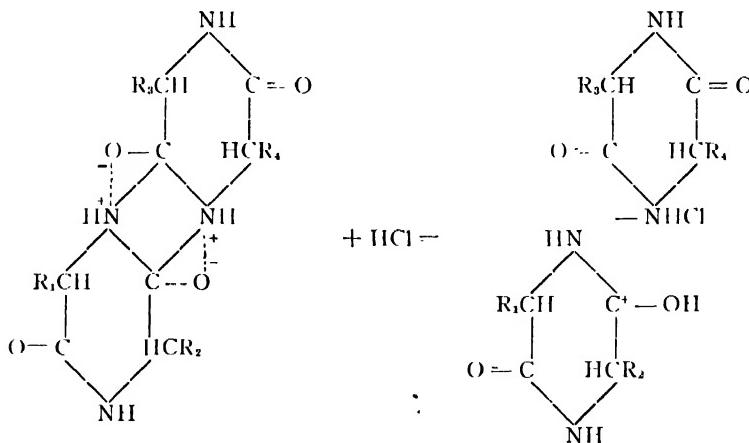
Since it would be a matter of chance which of the two nitrogen atoms in the linkage would unite with hydrochloric acid, the average composition of the protein components of the two ions must be identical. Similarly, the compounds with bases may either yield protein ions and metal or other basic ions, or they may yield two protein ions of identical average composition excepting that in one of them the metal ion is bound by covalency bonds and is therefore non-dissociable. If ionization occurred in the first of these two ways

then the addition of protein to a dilute solution of hydrochloric acid or sodium hydroxide should not reduce the concentration of chlorine ions in the one case or of sodium ions in the other. If, on the other hand, ionization occurs in the latter fashion described above then addition of protein to a solution of hydrochloric acid should reduce the concentration of hydrogen and chlorine ions equally, and addition of protein to a solution of sodium hydroxide should reduce the concentration of sodium and hydroxyl ions equally.

Now it appears that, in general, the hydrogen and chlorine ions are equally reduced in concentration by the addition of protein to solutions of hydrochloric acid. Thus Bugarszky and Liebermann [18] employed the potentiometric method, using the hydrogen electrode to determine the concentration of hydrogen ions and a calomel electrode to determine chlorine ions. The following data are compiled from their observations:

Egg albumin in 0.05 N HCl.

Grams Protein in 100 cc.	Per Cent of H^+ Bound by the Protein	Per Cent of Cl^- Bound by the Protein
0	0	0
0.4	9 0	10 7
0 8	18 9	20.2
1.6	33.3	38.0
3.2	60.2	64.0
6.4	96.6	76.0

These striking results have been confirmed by Rohonyi [114] employing egg albumin and albumose. The following were the results obtained, the substances being dissolved in 0.05 N HCl solution:

Substance	Per Cent	Per Cent of Chlorine Ions Bound	Per Cent of Hydrogen Ions Bound
Egg albumin	1.9	21.9	22.6
Albumose	1.8	37.2	35.9
Alanine	1.5	37.9	66.2

A decided difference between the modes of combination of proteins and an amino acid with hydrochloric acid is thus very clearly revealed.

Manabe and Matula [82] and Blasel and Matula [9] have shown that at low H^+ ion concentrations a greater proportion of H^+ is bound by serum albumin than Cl^-. Ringer [100] has confirmed this observation for albumoses, but he also finds that in higher concentrations of hydrochloric acid (1% of albumose in 0.1 N HCl) the H^+ and Cl^- are bound equally. The same tendency is shown in the observations of Bugarszky and Liebermann which are quoted above, for in the solution which contained the lowest proportion of hydrochloric acid to protein the Cl^- bound by the protein was about 20 per cent less than the proportion of H^+ which was bound.

On the other hand Loeb [81] finds that one per cent of originally isoelectric gelatin, when added to solutions of hydrochloric acid of pH varying between 1.4 and 2.7 causes no reduction of the PCl, the concentration of chlorine ions being the same in the gelatin hydrochloride solution as in the original solution of hydrochloride acid, although, of course, the concentration of hydrogen

ions is greatly reduced. Procter and Wilson also consider that gelatin hydrochloride is completely dissociated into gelatin ions and chlorine ions [97]. It therefore appears that either method of ionization may be exhibited by protein-acid compounds, the type of ionization depending possibly upon the type of protein or also, possibly, upon the circumstances which obtain at the moment of measurement. Among such circumstances may be included the presence of a membrane or other surface bounding the protein solution and separating it from another portion of the same solvent, which is permeable to inorganic ions but not to protein ions. For either only one of these methods of ionization is possible for a given protein, or else the two methods of ionization represent electronic tautomers which are in equilibrium with one another. In the latter event if a protein salt, yielding almost exclusively protein ions, were separated from another portion of the solvent by a membrane through which such ions cannot pass, an excess of osmotic pressure due to these ions would develop within the membrane. A reduction of this excess of pressure could be achieved in either of two ways, namely by the migration of diffusible electrolytes out of the membrane (the Donnan effect) or by the modification of the ionization of the protein in such a manner as to yield free inorganic ions and a single protein ion in place of two. The principle of LeChatelier would therefore lead us to anticipate that the equilibrium between the electronic tautomers would be so shifted as to increase the proportion of free inorganic ions yielded by the protein salt.

Turning, now, to the compounds which proteins form with bases, many facts are available which show that, as a general rule, they do not yield free metal ions. The observations of Galeotti [35], Bonamartini and Lombardi [10] and Rohmann and Hirchstein [113], which have been fully discussed elsewhere [111], have shown that the compounds which egg albumin forms with silver and copper, and the compound of casein with silver, do not yield metal ions in aqueous solution. An experiment which demonstrates in a very striking manner the fact that the compounds of casein with alkali metals do not split off the inorganic radical as an ion, is the following [111]: It will be recollected that casein, when deprived of its combined acid or base, is insoluble, and that if, to a solution of a caseinate of a base, exactly enough free acid is added (e.g., HCl) to completely neutralize the combined base, the free casein is entirely precipitated. Now one gram of ovomucoid [106] combines with 45×10^{-5} equivalents of HCl to form a compound such that less than 1 per cent of the acid remains uncombined (estimated by the hydrogen electrode). One gram of casein combines with 90×10^{-5} equivalents of KOH to form a compound such that less than $\frac{1}{2}$ of a per cent of the KOH remains uncombined [105]. If, now, these salts yielded an appreciable proportion of Cl^- and K^+ ions respectively then, on mixing two volumes of a solution of the ovomucoid salt with one volume of a solution of the casein salt (each of the same percentage concentration) the K^+ provided by the caseinate would be neutralized by the Cl^- provided by the ovomucoid salt and it might be anticipated that free uncombined casein would immediately be precipitated. Nothing of the sort occurs, however. If to 25 cc. of a 2 per cent solution of the casein salt are added 50 cc. of a 2 per cent solution of the ovomucoid salt, the mixture is no more opalescent than its constituent parts and *the conductivity of the mixture is the sum of the separate conductivities of the two protein salts.* If the mixture be allowed to stand at 36° C. in the presence of toluene to ensure sterility, after 24 to 45 hours a marked increase

of its opacity is observed; after two or three days traces of casein begin to be deposited, and after three to four days all of the casein is found to have been precipitated. The precipitation of the casein is accompanied by a marked increase in the conductivity of the mixture, attributable to the setting free of KCl. It is therefore evident that at the beginning the mixture must contain only minute traces of K^+ and Cl^- ions and that the protein salts only yield up these ions with extreme slowness.

The electrical conductivity of potassium caseinate in aqueous solution is not at all affected by the presence of an excess of the ions of the inorganic radical derived from potassium chloride [105], whereas if the caseinate dissociated potassium ions we would expect its dissociation to be diminished by the addition of KCl to the solution. We infer that potassium caseinate does not dissociate potassium ions in aqueous solution. Again, we may reach the same conclusion by quite a different process of reasoning and from very different experimental data. The conductivities of solutions of the caseinates and serum-globulinates of the alkaline earths do not decrease in direct proportion with dilution but somewhat more slowly, indicating a progressively increasing dissociation or, more correctly, "activity" of the caseinate on dilution [8]. The curve expressing the relation between equivalent conductivity (calculated on the basis of the inorganic radical) and the dilution is of the ordinary form [111] and from it we can, by exterpolation, estimate the maximum equivalent conductivity, i.e., the equivalent conductivity at infinite dilution of the salt; this we can do rather accurately since at readily attainable dilutions the conductivity already increases very slowly with dilution and obviously tends to approach a constant maximum. Now, as is well known, this maximum bears a constant proportion to *the sum of the equivalent conductivities of the ions into which the salt dissociates*. If the inorganic radical is dissociated as such, therefore, the equivalent conductivity of these salts cannot be less than that of the inorganic radical itself, but must exceed it by a quantity equal to the equivalent conductivity of the protein ion   In the following table are compared the observed equivalent conductivities (at infinite dilution) of a number of casein salts and those of the inorganic radicals which they contain, calculated from the data given by Kohlrausch and Holborn.[8]

It is evident that the equivalent conductivities of these protein salts are either equal to or substantially less than the equivalent conductiviy of the inorganic ion alone. On the supposition that the protein salt splits off the inorganic radical as an ion, not only the inorganic radical but also the protein must be participating in the conduction of electricity through its solution, and its equivalent conductivity, when completely dissociated, must be greater than that of the inorganic radical by the amount contributed by the protein ion. Hence these salts of casein and serum globulin cannot split off the inorganic radical to any important extent.

Two alternative possibilities need only be mentioned to be dismissed. The one is that the inorganic ion is dissociated as such but that its motion through the fluid under a potential gradient is hampered by the viscosity of the protein solution. As we shall see, however, the viscosity of protein solutions is not of this character and, in fact, the migration-velocities of inorganic ions

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[8] The migration-velocities of the ions at 18 degrees increased by 2 per cent per degree to reduce to the temperature employed and multiplied by the proportionality between ionic velocity and reciprocal ohms per cc., viz., 96.44.

Salt	Temperature, Degrees C.	Equivalent Conductivity at Infinite Dilution, Equivalent Concentration Taken as that of the Inorganic Radical. Reciprocal Ohms per cc. per Equivalent per Liter	Equivalent Conductivity at Infinite Dilution of the Inorganic Radical. Reciprocal Ohms per cc. per Equivalent per Liter
Sodium Caseinate (80×10^5 equivs. per gram)	25	63.5×10^{-3}	50.6×10^{-3}
Ammonium Caseinate (80×10^{-5} equivs per gram)	25	79.4×10^{-3}	73.2×10^{-3}
Potassium Caseinate (80×10^5 equivs per gram)	30	80.6×10^{-3}	81.0×10^{-3}
Calcium Caseinate (80×10^{-5} equivs. per gram)	30	35.9×10^{-3}	65.7×10^{-3}
Strontium Caseinate (80×10^{-5} equivs per gram)	30	30.7×10^{-3}	67.0×10^{-3}
Barium Caseinate (80×10^{-5} equivs. per gram)	30	42.1×10^{-3}	71.1×10^{-3}
Potassium Serum-Globulinate (20×10^{-5} equivs. per gram)	30	51.0×10^{-3}	81.0×10^{-3}
Calcium Serum-Globulinate (20×10^{-5} equivs. per gram)	30	23.5×10^{-3}	65.7×10^{-3}
Strontium Serum-Globulinate (20×10^{-5} equivs. per gram)	30	27.5×10^{-3}	67.0×10^{-3}
Barium Serum-Globulinate (20×10^{-5} equivs. per gram)	30	23.4×10^{-3}	71.1×10^{-3}

in protein solutions, or even in protein jellies, are not appreciably less than they are in distilled water [27]. It might also be supposed that these protein salts are not electrolytically dissociated at all but that they undergo hydrolytic dissociation and that the conduction of the current is really due to the free inorganic base. Solutions of the caseinates containing 80×10^{-5} equivalents of inorganic base per gram are, however, neutral to phenolphthalein and the concentration of free base in these solutions, determined by the hydrogen electrode, did not exceed 1.5×10^{-6} equivalents per liter (pH = 8.1 to 8.3). This quantity of free base would be inadequate to account for more than one-tenth of 1 per cent of the observed conductivity of a 2 per cent solution of potassium caseinate containing 80×10^{-5} equivalents of potassium per gram of casein. Solutions of serum globulinates containing 20×10^{-5} equivalents of inorganic base per gram are also neutral to phenolphthalein (pH 8.1 to 8.3) and the unneutralized base in these solutions would similarly be inadequate to account for more than a fraction of 1 per cent of the observed conductivities. Moreover, solutions of the caseinates of the alkalis and alkaline earths can be obtained which are neutral (50×10^{-5} equivalents of base per gram of casein) and these solutions therefore contain no free base; nevertheless they are excellent conductors of electricity. Thus a 2 per cent solution of potassium caseinate which is neutral to litmus possesses a conductivity of 92.7×10^{-3} reciprocal ohms per equivalent of base neutralized at 30° C. That this conductivity is not attributable to associated impurities, inorganic or other, is shown by the following facts:

(I) It bears a definite relation to the amount of base neutralized by the protein.

(II) The conduction of electricity is accompanied by migration of the casein to the anode, and the amount of casein transported to the anode is directly proportional to the quantity of electricity which is transported through the solution (Cf. below).

Similarly, in the case of the compound of egg-albumin with hydrochloric acid, Sjöquist showed, in one of the earliest researches in which modern physico-chemical methods were employed in the investigation of proteins [121], that if egg-albumin be dissolved in dilute hydrochloric acid, as the concentration of albumin is increased, keeping that of the HCl constant, the molecular conductivity (calculated for the concentration of HCl employed) diminishes until it reaches a constant minimum value, which is attained when about four grams are dissolved in 100 cc. of 0.025 N HCl. Now the above-quoted results of Bugarszky and Liebermann subsequently revealed the fact that, in this solution, at least 97 per cent of the hydrochloric acid is bound by the egg-albumin. The observed "molecular" conductivity (67×10^3) is at least 7 times greater than could be accounted for by the maximum possible residuum of unneutralized hydrochloric acid and must therefore have been due to the protein-acid compound.

We are therefore impelled to conclude that the compounds of proteins with acids and alkalis are ionized in aqueous solutions, but that in many cases, for instance the caseinates and serum globulinates of the alkalis and alkaline earths, and the compound of egg-albumin or of albumose with hydrochloric acid, the inorganic ion in not dissociated as such. The ions which are actually present in these solutions must therefore be protein ions, a proportion of which carry the inorganic radical bound in a non-dissociable form.

6. THE VALENCY OF PROTEIN IONS

If the salts which proteins form with inorganic acids and bases split off the inorganic ions as such, then the valency of the protein ion must be equal to the number of equivalents of the inorganic ion which is split off from every gram-molecule of protein. If, on the other hand, protein ions are formed in accordance with the equations:
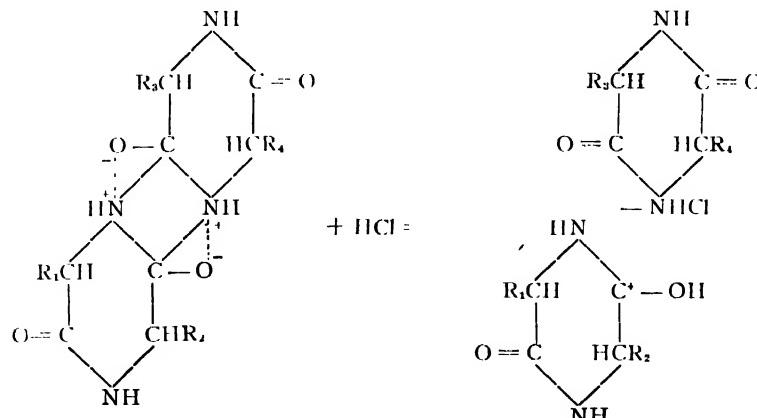
50×10^{-5} Equivalents of KOH per Gram of Casein (pH = 7.2).

Per Cent of Casein	Current in Amperes	Time of Passage	Electrochemical Equivalent in Grams per Coulomb
6	11.51×10^{-4}	2 hrs. 15 min.	0.0244 ± 0.0019
4	9.47×10^{-4}	2 hrs. 0 min.	0.0257 ± 0.0026
4	18.05×10^{-4}	2 hrs. 15 min.	0.0269 ± 0.0012
3	10.54×10^{-4}	2 hrs. 0 min.	0.0253 ± 0.0023
3	17.36×10^{-4}	2 hrs. 0 min.	0.0259 ± 0.0014
2	11.22×10^{-4}	2 hrs. 0 min.	0.0238 ± 0.0022
2	15.85×10^{4}	1 hr. 25 min.	0.0219 ± 0.0022
		Average	0.0248 ± 0.0020

80×10^{-5} Equivalents of KOH per Gram of Casein (pH = 8.2).

Per Cent of Casein	Current in Amperes	Time of Passage	Electrochemical Equivalent in Grams per Coulomb
3.75	10.82×10^{-4}	2 hrs.	0.0259 ± 0.0022
3.75	16.03×10^{-4}	2 hrs.	0.0222 ± 0.0015
3.75	8.01×10^{4}	4 hrs.	0.0250 ± 0.0015
3.75	8.74×10^{-4}	4 hrs.	0.0282 ± 0.0014
3.75	18.31×10^{-4}	2 hrs.	0.0194 ± 0.0013
3.75	10.56×10^{-4}	2 hrs.	0.0209 ± 0.0023
3.75	19.50×10^{-4}	1 hr.	0.0230 ± 0.0025
		Average	0.0235 ± 0.0018

100×10^{-5} Equivalents of KOH per Gram of Casein (pH = 9.3).

Per Cent of Casein	Current in Amperes	Time of Passage	Electrochemical Equivalent in Grams per Coulomb
3	2.03×10^{-4}	2 hrs.	0.0226 ± 0.0020
2	12.56×10^{-4}	1 hr.	0.0217 ± 0.0039
		Average	0.0222 ± 0.0030

of base to casein in this film falls to that which obtains at "saturation" of the base with casein. Any additional casein thus migrating into the film in contact with the anode must be precipitated as uncombined casein. The cations, containing the potassium, migrate to the cathode and there react with part, part with two charges and change their sign, migrating thereafter to the anode. Hence the electrochemical equivalent which is actually measured in solutions of all reactions is that of casein at "saturation" of the base with protein.

Rejecting the data obtained in solutions *alkaline* to phenolphthalein (that is, in those solutions containing 100×10^{-5} equivalents of base per gram) on account of the possible error arising from hydrolysis of the protein due to the ten-thousandth molecular excess of alkali which these solutions contained, the average of all the determinations yields the value of 0.0242 ± 0.0019 for the electrochemical equivalent of casein.

Multiplying this by the Faraday constant, 96,530, we obtain the weight of casein in grams which transports one atomic charge. This is 2336 ± 183.

Now at "saturation" of a base by casein, the proportion of base to casein is 11.4 x 10^{-5} .equivalents per gram, corresponding, if at this reaction casein combines with only one molecule of base, with the molecular weight of 8772. If we assume that at "saturation" of the base with casein two, three, or four, etc., molecules of base are bound up in one molecule of caseinate, the molecular weight of the casein would be two, three or four, etc., times 8772. Either of two suppositions may now be entertained:

(I) The potassium caseinate dissociates into potassium and casein ions. If this be the case then the weight of the casein anion must be that of the molecule of casein, i.e., a multiple of 8772, and the *valency* of the casein ions must be a multiple of:

$$\frac{8772}{2336 \pm 183}$$

i.e., of 4.8 ± 0.3 or, in round numbers, 4. It would follow from this that four potassium ions must be split off by casein when combined with the smallest proportion of potassium hydroxide which yields a soluble compound and hence this compound must arise from the union of four molecules of potassium hydroxide with one molecule of casein. But the *maximal* combining capacity of casein for potassium hydroxide, estimated by the hydrogen electrode, is 180 x 10^{-5} equivalents per gram. The number of molecules of potassium hydroxide combined with one molecule of casein to form this compound must therefore be $\frac{180}{11.4} = 16$ times as great as the number combined with casein to form the compound which contains the minimal proportion of potassium hydroxide. If this latter compound results from the union of four molecules of potassium hydroxide with one of casein, then the compound containing the maximal proportion of base must arise from the union of 16 x 4 = 64 molecules of base with one molecule of casein. It will hardly be supposed that one molecule of casein weighing 8772 contains 64 points at which a base may unite with it. The casein anion as it actually exists in this solution, prior to its migration into the film in immediate contact with the anode, must in that event be 64-valent, and a 64-valent ion in aqueous solution is something entirely beyond the boundaries of our experience. The alternative to these inadmissible deductions is the following:

(II) The potassium caseinate dissociates into two protein ions of approximately equal weight. If this be the case then the weight of the casein anion must be *half* that of the molecule of casein, i.e., 4386, and the *valency* of the casein ions must be $\frac{4386}{2336 \pm 183} = 1.9 \pm 0.15$, or, in round numbers, 2. Now this is exactly the effective valency which, as we inferred above, protein ions must possess if they are formed by the splitting of unions between diketopiperazine rings and undergo decomposition at the electrodes with liberation of the inorganic radical and change of sign.

Another consequence which follows from this conception of the ionization of the caseinates of the alkalis is that during electrolysis the loss of casein from the solution in the anodal region should be double the loss experienced in the cathodal region, for, as we have seen, in the anodal region two ions combine to regenerate free casein which is precipitated, in the cathodal region during the same interval ,of time *one* ion changes sign and migrates back to the anode. It might be imagined that this ion would simply replace one of

hence the viscosity of water is increased by substances which are dissolved therein and possess molecules which are impenetrable by and considerably larger than those of water itself.

It has been shown by Einstein [28, 30] that the viscosities of very dilute solutions of this character may be expressed as a linear function of the relative volumes occupied by the solute in the solution as follows:

$$\eta = \eta_0(1 + 2.5\,\varphi)$$

where η is the viscosity of the solution, η_0 that of the solvent and φ is the fraction of the volume of the solution which is occupied by the dissolved substance. In more concentrated solutions this relation fails to apply because the free motion of the water molecules in such solution may be simultaneously constrained by several suspended particles. It has been shown by Arrhenius [5], however, that a more general relation:

$$\frac{\eta}{\eta_0} = A^n$$

can be deduced from Einstein's formula and applied to solutions of high concentration, η being, as before, the viscosity of the solution, η_0 that of the solvent, n the concentration of the solute and A a constant which differs with differing solvents and solutes but is always the same for solutions of differing concentration of a given solute in a given solvent.

This formula was applied to solutions of sodium caseinate containing 80 x 10^{-5} equivalents of sodium per gram) by Sackur [115], with the following results:

N (in Equivalents of Sodium)	$\frac{\eta}{\eta_0}$ (15° Centigrade)	Log A
0.01830	1.870	14.8
0.01370	1.581	14.5
0.00915	1.363	14.3
0.00547	1.202	14.6
0.00458	1.165	14.5

and its applicability to solutions of gelatin and casein hydrochloride has recently been affirmed by J. Loeb [81].

A remarkable feature of Sakur's results is the extraordinarily high value of A, involving an unusually rapid increase in viscosity with increasing concentration. For the majority of crystalloids the value of A is not greatly in excess of unity, while for sodium caseinate it is of the order of 10^{14}. This fact alone would lead us to suspect that the mechanism which produces the viscosity of these solutions is different in nature from that which produces the viscosity of solutions of crystalloids. Sackur has endeavored to ascertain which constituent of the solutions of sodium caseinate plays the greater part in determining their viscosity. He arrived at a conclusion the correctness of which more recent investigations have fully established, by a process of reasoning, however, which recent investigations have shown to be in some respects fallacious. He argued that the viscosity of these solutions might be attributable, primarily, to undissociated sodium caseinate or the product of its hydrolytic dissociation,[10] i.e., free casein, or to caseinate ions. Hydrolytic

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[10] Since free casein is insoluble this possibility may be dismissed.

dissociation would, he believed, be diminished by the addition of alkali and increased by the addition of acid. In the former case, according to his view, the number of caseinate ions should be increased, in the latter decreased. He found that the addition of alkali *increased* and the addition of acid *diminished* the viscosity of a solution of sodium caseinate which was initially neutral to phenolphthalein (pH = 8.2); the following are among his results:

Per Cent of Casein	Normality of Total Sodium	$\frac{\eta}{\eta_0}$ (15° Centigrade)
0.716	0.0063	1.24
0.716	0.0126	1.36
0.716	0.0189	1.38
0.716	0.0252	1.34

hence, he argued, the viscosity of these solutions is primarily attributable to *caseinate ions*.

We have seen, however, that the caseinates of the alkalis do not undergo hydrolytic dissociation in solution. In fact, the initial solution to which Sackur added alkali in order to *suppress* hydrolytic dissociation was neutral to phenolphthalein and therefore could not have contained more than 10^{-5} normal NaOH derived from hydrolytic dissociation of the sodium caseinate. We have also seen that on adding alkali to a solution which is neutral to phenolphthalein the added alkali does not remain unneutralized, as Sackur assumes, but, on the contrary, is partially bound by the casein until 180×10^{-5} equivalents are combined with every gram of casein. But we have also seen that each successive equivalent of combined alkali opens out a pair of bonds between diketopiperazine rings in the protein molecule, giving rise to another pair of ions. With increasing content of combined base, therefore, the caseinate, apart from slight modification of its degree of dissociation, yields a corresponding proportion of ions. The fact that the viscosity of caseinate solutions increases with the alkalinity is therefore in strong support of Sackur's thesis that the viscosity of these solutions is primarily attributable to protein ions, although not for the reasons which he advances. The fact that the increase in viscosity with increasing alkalinity attains a maximum at just about the same time that the combining capacity of the casein attains a maximum lends further support to this hypothesis.

The view that the viscosity of protein solutions is in a remarkably high degree dependent upon the protein ions which they contain has also been advanced, with substantial experimental support, by W. B. Hardy [44] and by Bottazzi * [15]. The latter observer has shown that the viscosity of proteins is at a minimum when ionic protein is absent, that is, when the protein is "isoelectric," and that on adding either acids or bases to this solution the viscosity increases. Loeb has confirmed these results [81].

It can readily be shown that the viscosity of protein solutions is of an altogether different type to the viscosity, for example, of solutions of sugar or glycerol in water to which Einstein's and Arrhenius' formulas also apply. It must be recollected, however, that these formulas must necessarily apply to any type of viscosity of which the local magnitude depends upon the relative volumes of the solute and solvent. Consequently these formulas do not enable us to distinguish between the usual type of viscosity and that arising from

* See paper by F. Bottazzi, this volume. *J. A.*

internal friction of any other origin, provided only, which is in any case extremely likely, that its local magnitude depends upon the volume of solute which is contained in unit volume of solution. In other words, these equations yield us no information concerning the *origin* of viscosity in a particular solution but merely inform us that its *magnitude* is proportional to the relative volume of the solute.

The extraordinary magnitude of A for solutions of the caseinates might conceivably arise from the relatively great proportion of the volume of the solution which is occupied by protein particles, although in that case we must further assume, as Loeb has done [81], the existence of an enormous galaxy of associated or occluded water molecules, forming a mass of a nature totally different from that originally contemplated by Einstein in the derivation of his formula.[11] But, quite apart from the magnitude of A, there is one remarkable characteristic of the viscosity of protein solutions of which the mere displacement of the molecules of the solvent by molecules or micellae of the solute affords no intelligible explanation. For whereas the ordinary type of viscosity hinders the motion of ions and the diffusion of crystalloids in direct proportion to its magnitude, the type of viscosity which is exhibited by solutions of proteins and some polysaccharides (such as agar) affords no hindrance, or at the most very slight hindrance, to the motion of ions or of crystalloidal molecules.

Thus it was originally pointed out by Graham [40, 41] that the velocity with which various crystalloids diffuse through gelatin jellies is remarkably near to that with which they diffuse through water, and Voightlander [136] has confirmed this result for agar jellies. Similar results have been obtained by Hufner [52]. According to Bechhold and Ziegler [7] the rate of diffusion of concentrated crystalloids is diminished by gelatin jellies, and the degree of hindrance is materially modified by the presence, within the jelly, of other dissolved substances, but the hindrance is extremely small in comparison with the enormous viscosity of the jellies.

Similarly Reformatzky [98] has shown that the velocity with which methyl acetate is decomposed by acids in agar jelly (i.e., the number of molecular collisions per second) is within 1 per cent of its value in pure water.

Lodge [79], Wetham [139, 140], and Masson [84] have shown that the specific mobilities of the majority of inorganic ions are the same in agar jellies as in water. Dumanski [27] has shown that if allowance is made for the diminution of the cross-section of the conducting field due to the presence of gelatin $\left[= \left(\frac{g}{c} \right) \right]$, where g is the number of grams of gelatin per gram of solution and c is the specific gravity of gelatin) the conductivities of inorganic salt solutions in gelatin jellies are very slightly less than those of equally concentrated solutions in pure water. The dependence of the conductivity of solutions of caseinates upon their dilution is of perfectly normal character and displays no influence of changing viscosity [111] although, as the above-cited measurements of Sackur reveal, the viscosity of these solutions varies enormously with their dilution.

On the other hand, the intimate dependence of the conductivity of solutions of electrolytes upon the ordinary type of viscosity has been commented

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[11] Because, being largely fluid, it would presumably be subject to deformation by strains, i.e. in consequence of its own motion relatively to the surrounding fluid. Moreover, interchange of water between the outside and the inside of the mass would permit penetration by the molecules which occupy the surrounding domain.

upon and quantitatively estimated by a host of observers, among whom Walden may be especially mentioned [137]. Viscosities, for less than those of the most dilute jellies, profoundly diminish the conductance of solutions of electrolytes. Nor must it be imagined that the viscosity of a protein jelly is essentially different, in any respect save magnitude, from that of a protein solution, for, as von Schroeder [117] has shown, the viscosity of a solution of gelatin, cooled below the gelation-point, increases progressively and regularly with time, until the extremely viscous solution passes insensibly into a jelly.

Not only inorganic but also protein ions are profoundly influenced in their mobilities by the type of viscousness which alcohol—or glycerol—water mixtures exhibit. Thus the author has shown [108] that the mobilities of caseinate ions in alcohol-water mixtures are almost exactly inversely proportional to the viscosity of the solvent, and this despite the fact that the viscosity of the solution, measured by the time it takes to run through an Ostwald viscometer, is in many cases so profoundly modified by the introduction of the caseinate itself as to be doubled in magnitude. This portion of the total viscosity may be neglected in estimating the effect of viscosity upon the conductivity of the mixture. Solutions of KCl in alcohol-water mixtures are, comparatively speaking, unaltered in their viscousness by the KCl itself, yet the dependence of the conductivities of solutions of potassium caseinate upon the percentage of alcohol which they contain (between 0 and 60%) obeys exactly the same law as that which applies to the conductivity of KCl solutions in alcohol-water mixture, a law which implicitly involves the conclusion that the total effect is due solely to the alteration of the mobilities of the ions attributable to the viscousness of the solvent. *In estimating the influence of viscosity upon the mobilities of ions we can entirely disregard the influence of the total viscosity of the solution which, even if comparable in magnitude with the viscosity of the solvent itself, is attributable to protein.*

Recollecting that the multipolar character of the protein molecule enables it to exercise a restraint upon the mobilities of adjacent bipole or multipole molecules, and that the great volume of the protein molecules brings the peripheries of these multipoles into unusual proximity with one another (in comparison with the distance separating the peripheries of much smaller molecules in like dilution), we are strongly impelled, by the facts outlined above, to the belief that the type of viscosity which solutions of proteins exhibit may owe its existence to an orientation of the molecules equivalent, in its resistance to deformation, to a net-structure extended in three dimensions. A structure of this character would offer no resistance to the passage through it of a quickly moving body which is smaller than its meshes, other than that due to the fact that the material which composes the net occupies a small fraction of the area which the body must traverse, but to any force which involves deformation of the structure, for instance, a force which seeks to drag it through a tube of small diameter, it will oppose a very considerable resistance. On the other hand, the resistance which is offered to a small moving body by a viscous liquid (viscous, that is, in the ordinary sense) is accurately measured by the resistance which the liquid offers to passage through a tube. Now the *direct* methods which we employ to measure the viscosity of fluids are all such (rotation of a relatively large body within the fluid; passage of the liquid through a capillary tube, etc.) as would involve *the deformation of any molecular structure within the fluid*. Our *direct* methods of estimating viscosity do not enable us to distinguish between that type of viscosity which is attributable to

a structure within the fluid and the other type of viscosity contemplated by Einstein in the derivation of his equation. The indirect measurement which the conductivity estimations afford, however, only reveals the latter type of viscosity and, as we have seen, when we employ this method of estimation the presence of protein is found to leave the viscosity of the solvent unaltered. It is, of course, not in the least necessary that the net-structure should form a physical continuum in order that it should display these properties. If we conceive it in the likeness of a tennis-net extended in three dimensions, the molecules of protein would be situated at the intersections of the threads forming the net and the threads stretching from one intersection to the next would be represented by lines of force (or Faraday-tubes).*

That this is the true origin of the major part of the viscosity of protein solutions has been demonstrated by Garrett [36]. He observed that the logarithmic decrement of a disc oscillating in gelatin or albumin solutions is not a constant, as it is in water or other homogeneous fluids, but, on the contrary, increases as a linear function of the time. This was traced to adhesion between the disc and the protein, since if the disc was taken out of the fluid and washed the initial value of the decrement was always the same. This is obviously to be explained, on the basis of the above hypothesis, by the adhesion of portions of the net-structure to the oscillating disc. Garnett has further observed that very slight mechanical disturbance of a gelatin-solution produces considerable alteration in the magnitude of the oscillation-decrement.

It remains for us to consider somewhat more particularly how this net-like structure arises in solutions of proteins.

We have seen that isoelectric protein contains a number of unions between diketopiperazine rings in which residual valencies are chiefly neutralized internally. In the presence of any solute containing actual or potential ions, however, these residual valencies must, in greater or less proportion, turn outwards and enter into electrostatic equilibrium with ions in the solvent. In particular, adjacent protein molecules must tend to assume a definite orientation with respect to one another, which may be represented thus:
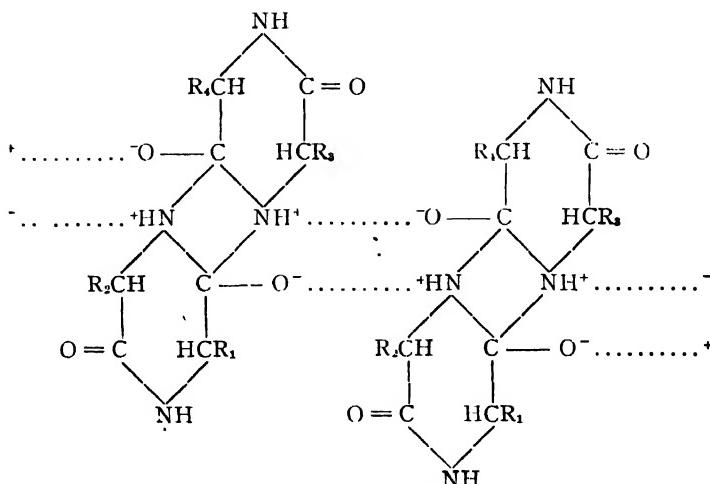
In consequence of the formation of aggregates of this type, many proteins (globulins, for example) are insoluble at the isoelectric point. In solution the effect of their existence must be to impose a resistance of electrostatic origin to any deformation of the fluid which involves relative displacement of the protein molecules. Since, in isoelectric protein, the majority of these valencies are neutralized internally, the viscosity of electrostatic origin must be at a minimum in such solutions. In the presence of acids or bases, however, protein ions are formed, and an electrostatic viscosity of much greater magnitude now comes into play, because every bond in the protein molecule which has been opened up by the neutralized acid or base now contributes its quota to the total effect, instead of a small proportion of these bonds, as in solutions of isoelectric protein.

The existence of this type of viscosity in ionised solutions was first pointed out by Sutherland [125]. He explains the method by which it arises in the following words: "In an electrolytic solution the electric forces acting amongst the ions introduce powerful stresses and, in association with them, important viscosities of an interesting type. As the solvent in ionizing the solute pulls the ions of the molecule asunder against their strong electric attraction, we must suppose that in general it keeps the positive ion as far as possible from its nearest negative neighbors. Thus the positive and negative ions are uniformly distributed through the solvent, which preserves the average uniformity in such a way that each of 2q ions in a volume of 1 is at the centre of a domain $\frac{1}{2q}$, and the domains are arranged in regular order, those of positive and negative ions occurring alternatively. In this way an ionized solution is the seat of a powerful distribution of polarity similar to that which I have taken to be the basis of rigidity."

Einstein similarly refers rigidity to polar forces operating between adjacent molecules [29]. Since viscosity is actually rigidity of instantaneous duration, its origin must be the same as that of rigidity in solids. The type of viscosity contemplated in the derivation of Einstein's formula is attributable to polar forces operating between adjacent molecules of the solvent or molecules of the solvent and those of the solute. The type of viscosity depicted by Sutherland originates in the polar attractions or repulsions (as modified by the solvent) of the molecules or ions of the solute for one another. If we imagine the solvent removed from the solution while retaining its effect (due to its dielectric capacity) upon the distribution of the particles of the solute, then the viscosity of this residue would be essentially similar in origin to that of the solvent itself. It is obvious, therefore, that this type of viscosity must also vary with the volume of the solute in the sense required by the formulas of Einstein and Arrhenius.*

That this viscosity of electrostatic origin would correspond exactly with the viscosity displayed by protein solutions is evident, but the inquiry may suggest itself why this type of viscosity has not previously been detected in solutions of other electrolytes. The reply is that the great volume of protein molecules and ions brings their peripheries much closer together in any given space-distribution than the peripheries of smaller molecules or ions which are similarly dispersed, and since these polar attractions fall off in intensity very rapidly as the distance between the polar bodies increases, it follows that in solutions of ordinary electrolytes this type of viscosity must be evanescent

* See also papers on viscosity, plasticity, gel structure, etc., in Vol. I of this series. *J. A.*

in comparison with its magnitude in protein solutions. The lines of force connecting the uniformly distributed ions will be much longer and in proportion to the square or some higher power of this distance they will be weaker. As it has elsewhere been expressed "The fact that the viscosities of solutions of crystalloid electrolytes have not so far revealed the presence of a net-structure within them is possibly attributable to the tenuity of the net and the fineness of its framework; to revert to the analogy employed above, a net of the finest and most flexible silk will readily pass without appreciable resistance through a tube which would offer a considerable resistance to the passage of a net of coarse thread. The phenomena to which I have drawn attention may quite conceivably be of very general occurrence, and of greater physical magnitude in solutions of proteins simply because of the greater size of the protein molecules" [111, p. 326]. As a matter of fact, however, it is really evidenced in the effect of dilution upon the conductivity of electrolytes. The conception of electrostatic viscosity, introduced by Sutherland, led him independently to the modern view of electrolytic dissociation [8, 37, 85] which considers that electrolytes are completely dissociated in solution, the variation of conductivity with dilution being due to the increase of the "activity" of the electrolyte, which Sutherland regards as attributable to the weakening of electrostatic attractions by the wider dispersion of the polar particles. Since the electrostatic viscosity of protein solutions is, as we have seen, exceedingly high, it might be imagined that the effect of dilution upon their equivalent conductivity should also be exceptionally great, whereas, as a matter of fact, the effect of dilution upon the equivalent conductivities of caseinates and serum globulinates of the alkalies is comparatively small. It must be recollected, however, that the effect of electrostatic viscosity appears in two ways in the customary dilution-formula for conductivity, namely in the magnitude of the "dissociation-constant" and also in the magnitude of the specific equivalent mobilities of the ions. In solutions of the caseinates and serum globulinates of the alkalies it has been shown that the specific equivalent mobilities of the protein ions are about one-half the magnitude of the mobilities of the crystalloidal ions which are combined with them and approximate to the constant minimal magnitude of the specific mobilities of heavy ions [17].

POSTSCRIPT

The above chapter was written at the end of 1923. Very considerable developments have occurred in certain aspects of protein chemistry since that date, but no attempt has been made to incorporate these in the chapter because nothing has been discovered which necessitates the modification of the views therein set forth. On the contrary, the existence of Azine structures in protein molecules, which was at that time inferred, on the one hand from the susceptibility of proteins to hydrolysis by proteolytic enzymes and the affinity of these enzymes for Azine structures, and on the other hand from the physico-chemical behavior of the salts of proteins with acids and bases, has now, thanks to the discoveries of Abderhalden, Ssadikow and Zelinsky [143] and Levene and co-workers,[144] been established upon a firm basis. The time is, in fact, rapidly approaching when every investigator in this field will recognise, as the author recognised in 1911,[145] that the assumption of an amino-acid structure of protein is totally inadequate to account for the facts which accompany protein self-formation. It is essential to invoke also the

aid of internal amphoteric structures which, as we now know, are azine rings.

So far as the author is aware no substantial advance in our knowledge of viscosity in protein solutions has occurred since the foregoing chapter was written. That problem remains, therefore, in the condition in which it was then depicted.

Adelaide, South Australia,
    July, 1927.

of the percentage of gelatoses or of gelatones in the purified product, nor is the jelly-strength or mutarotation (after C. R. Smith) given, from which a rough idea of their percentage might be formed. The gelatin apparently lost 20 to 25 per cent during purification, which introduced a like error into some of Loeb's earlier experiments. [See, e.g., Harvey Society address, *Science* (N.S.), **52,** 451 (1920); *J. Gen. Physiol.,* **3,** 89 (1920); also the present book (p. 58).]

Again Loeb has failed in each case to define exactly the ash and moisture content of the gelatin used, both being essential (especially the latter) in fixing the percentage of gelatin in solution. C. R. Smith [*J. Am. Chem. Soc.,* **43,** 135 (1921)] and Miss A. M. Field [*ibid.,* **43,** 667 (1921)], had shown how to prepare ash-free gelatin, which is by no means identical with isoelectric gelatin. Loeb gives (p. 35) "a result of an ash determination made by Dr. D. L. Hitchcock on a sample of gelatin selected at random" from one of Loeb's stock solutions containing 12.69 per cent gelatin. The analysis showed about 0.1 per cent ash, apparently $Ca_3(PO_4)_2$. Loeb says this amount of ash is without influence, but since C. R. Smith has shown that Ca may exert a powerful influence, it would have been better had Loeb determined the ash of *every* specimen of gelatin he actually used. The paper of Oakes and Davis [*J. Ind. Eng. Chem.,* **14,** 706 (1922)] also shows the importance of "ash."

Loeb's experimental results are reported mainly in curves or graphs which your reviewer believes are incorrectly plotted and drawn. This is quite evident in the graphs of Chapter VI, where equal increments on the axis of abscissas are given M/2, M/4, M/8, M/16, M/32, etc.

(*The substance of some paragraphs omitted here is given at the end of the paper by Wherry in this volume.*)

Therefore values of about pH 2 to pH 3 represent a very high acidity. Curves which Loeb shows with equal branches, resembling parabolas, should rise sharply, almost asymptotic to the pH 4.7 ordinate, and after turning, gradually approach to axis of abscissas. Some other points may be epitomized as follows:

The view (Chapter II) that proteins fix acids by their free NH_2 groups and acids by their free $COOH$ groups, while simple, does not accord with the observation that deaminized gelatin (Blasel and Matula)* and highly hydrolyzed gelatin (T. B. Robertson) both fix about as much acid as the original gelatin. Jordan Lloyd and Mayes [*Proc. Roy. Soc.,* B. **93,** 69 (1922)] give additional evidence that disproves this appealing but incorrect chemical explanation.

Loeb's interesting qualitative experiments (Chap. II) show, as Bancroft remarked, that acid or plus-charged gelatin (pH less than 4.7) fixes only anions, while alkaline or minus-charged gelatin (pH greater than 4.7) will fix only cations, which is only to be expected. Clay or fine silica might act similarly.

His quantitative experiments (Chap. IV) show, e.g., that the number of cc of different N/10 acids required to bring isoelectric gelatin to the same pH varies roughly in accordance with the pH (available or effective acidity) of each acid at that pH. Thus at pH 3 the effective acidity of HCl and H_2SO_4, oxalic, and phosphoric acids are about 3, 2 and 1 respectively, and Loeb found that to bring one gram of isoelectric gelatin to pH 3, it took (in cc. of N/10 acids) 7.2 HCl, 7.5 H_2SO_4, 13.15 oxalic, 20.7 phosphoric. Most of his experiments stop at about pH 2, which represents an acidity equal only to N/100 HCl (pH 2.02).

Assuming that deviation from the curves is due only to experimental errors, these results, while not inconsistent with the view that chemical compounds have been formed, may simply be due to the fact that within the pH range of the experiments,† gelatin has, at each pH, a more or less definite free adsorptive surface and adsorbs acids in proportion to their free field of force. The fact that material chemical changes (see above) in gelatin do not affect its acid combining capacity appreciably favors the adsorption rather than the chemical view. Even W. B. Hardy (*see* Loeb, p. 9), who latterly expressed belief in salt formation, stated that the reactions are not precise. No one has ever prepared chemically pure gelatin, and estimates of its combining weight vary from about 768 (Proctor and Wilson) to about 96,000 (C. A. Smith). Jordan Lloyd gives chemical evidence that it cannot be less than about 10,300 and Loeb apparently believes it to be between 12,000 and 25,000. As Hardy indicates, it depends upon conditions, and your reviewer believes it will also vary with the kind of gelatin. It is

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* Since this was written D. I. Hitchcock has published opposing evidence.

† The work of Hoffman & Gortner (see Second Colloid Symposium Monograph, 1925) has since shown that beyond the pH limits used by Loeb, the experimental results and the necessary conclusions are quite different from what Loeb found.

still to be demonstrated that these compounds possess the definiteness which is at present connoted by the expression "chemical compound."

The pH (that is, the effective reaction) of acids is controlled by the specific nature of their anions; the pH of alkalis by the specific nature of their cations. The pH of $N/100$ HCl is 2.02, but the pH of $N/100$ acetic acid is 3.37. Why? Because in aqueous solution Cl releases H + more readily than does CH_3COO —. This means that HCl is a strong acid and acetic acid a weaker one.

Hofmeister, Ostwald and M. H. Fischer observed the effect on proteins, of equal molar concentrations of various acids and alkalis and then compared the consequences of their differing pH or effective reaction. Loeb, on the other hand, uses enough of various acids and alkalis to make their effective reactions (pH) equal and then compares the varying quantities required. What he considers "practical identity"—e.g., in curves of Figures 19 and 20 (pp. 79 and 80)—may appear differently if the curves are correctly plotted. He even comments (p. 80) on the anomalous behavior of acetic acid, probably caused by sol formation (see below).

That the problem of the swelling of gelatin in acids is not as simple as a Donnan equilibrium formula would indicate is evident from the work of A. Kuhn * [*Kolloidchem. Beihefte*, **14**, 202 (1921)], who found it to be dependent upon four factors:

A (1) Hydration (simple swelling)
B (2) Sol formation (incidental peptization)
   (3) Hydrolysis
C (4) Dehydration (flocculation)

The maximum is reached when, with increasing concentration of acid, hydration is overbalanced by sol formation and hydrolysis. Probably it was sol formation that caused Loeb to lose about 20 per cent of his gelatin in his earlier experiments (p. 58). Since sols (as Graham distinctly pointed out) do diffuse, albeit though slowly, we have here another factor working contrary to the Donnan equilibrium. Experimenting with 50 organic acids, Kuhn could not decide if the combination was chemical or adsorption.

Loeb argues (p. 16) that "if the addition of a salt to a protein solution diminishes its osmotic pressure by causing an increased formation of aggregates, the same addition of salt should increase the viscosity of such a solution. The reverse, however, happens, the viscosity of the solution being decreased by the addition of salt." Loeb here entirely overlooks the existence of the zone of maximum degree of colloidality. [See *J. Am. Chem. Soc.*, **43**, 434 (1920).]† Viscosity does often increase as particles aggregate, as in cooling gelatin solutions, but viscosity may also increase as particles are dispersed, as when cream is homogenized, or karaya gum dispersed.

Loeb makes the sweeping assertion (p. 278) that "there is only one source of colloidal behavior - namely, the Donnan equilibrium,—at least as far as the proteins are concerned." Donnan can hardly believe this, for Loeb quotes him (p. 22) as saying [*J. Chem. Soc*, **105**, 1963 (1914)] that in the comparatively simple case of a copper ferrocyanide membrane and potassium ferrocyanide solutions, "the phenomena are not so simple as supposed in the theory." The great danger of applying mathematics to chemical and physical problems lies in the fact that we may be blinded by the logical perfection of this mere tool and make erroneous assumptions, or else neglect important factors which so often crop up unexpectedly in nature. The fact that some of the assumptions involved in Donnan's equations do not apply to gelatin has been pointed out by Jordan Lloyd—e.g., the gelatin "ion" does diffuse. The basic assumption that a true hydrolyzable "salt" is formed is, to say the least, doubtful.

A word in defense of Thomas Graham is necessary, for his classic papers are seldom consulted in the original. Loeb states (p. 275): "Graham had suggested the distinction between colloidal and crystalloidal substances, but it was found later that one and the same substance—e.g., NaCl, may behave when in solution either as a crystalloid or a colloid. It was then proposed to drop the distinction between colloidal and crystalloidal substances, and distinguish between the colloidal and crystalloidal state of matter." This is surprising, for Loeb on page 1 quotes from Graham the very paragraph wherein the word "colloid" was born; the particular sentences of Graham are: "As gelatine appears to be its type, it is proposed to designate substances of the class as colloids and to speak of their peculiar form of aggregation as the colloidal condition of matter. Opposed

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* Some of Loeb's views in opposition to Kuhn are given in the Appendix, which is a reprint of one of Loeb's papers.
† See also Chapter I in Vol. 1 of this series.

to the colloidal is the crystalloidal condition. Substances affecting the latter form will be classed as crystalloids. The distinction is no doubt one of intimate molecular constitution." Further on in the same paper, "Liquid Diffusion Applied to Analysis" (1861), Graham says: "The mineral forms of silicic acid, such as flint deposited from water, are often found to have passed during the geological ages of their existence from the vitreous or colloidal into the crystalline condition (H. Rose). The colloidal is in fact a dynamical state of matter, the crystalloidal being the statical condition." And still further: "The inquiry suggests itself whether the colloid molecule may not be constituted by the grouping together of a number of smaller crystalloidal molecules and whether the basis of colloidality may not really be due to this composite character of the molecule." So Graham did know that the same substance can exist in both colloidal and in crystalloidal state. Modern research has fixed dispersion into particles between about 100 mμ and 5 mμ as the criterion of the colloidal condition, and the particles may be crystalline or consist of random clusters. But these statements of Graham still hold true.

Physico-Chemical Investigations on the Heat-Denaturization of Proteins

By Prof. Dr. Mona Spiegel-Adolf,[*]

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Among physical influences which are capable of inducing changes in proteins because of their biological and practical significance, most attention has been given those which follow a rise in temperature. Nevertheless, until the present, there has existed no single generally accepted explanation of the changes brought about by heat on the protein molecule, or of the conditions of their origin. As criteria of the heat-change, we usually find given irreversibility and decreased solubility.

A physico-chemical investigation can serve only indirectly to clarify the problem of the chemical basis of the heat-changes of protein. This can be accomplished by comparing the properties of the product of heat precipitation with those of true proteins and proteins that have been modified by other agents. Besides, this method might be especially suitable for the study of the conditions necessary to initiate thermal changes.

Accordingly these two questions will be the principal topics of this paper, in which are presented a series of my own studies,[1] including data as yet unpublished concerning the heat changes and other forms of denaturization of protein. The experiments were carried out with the two water-soluble proteins, serum- and egg-albumin, as well as with the water-insoluble globulin and casein.

I

Before progress could be made in the investigation of heat-denatured proteins, it was first necessary to determine the conditions of their formation. The experiments with water-soluble proteins were designed to bring about as complete a heat-precipitation as possible, in order to avoid the necessity of closer examination of a possible heat-fractionation of the albumin (cf. Halliburton [2]), and also in order that the original and the altered products might be comparable in composition.

Now the occurrence of heat-precipitation generally appears to be ultimately connected with the presence of electrolytes, and from the writer's own experience, these also influence the composition of the coagulum which forms. Therefore Cohnheim's [3] attempt to induce coagulation of soluble proteins which have been freed by thorough dialysis of salt and alkali, seemed to be entirely justified. Although it is said that heat will precipitate serum-albumin

[1] Kolloid-Z., 35, 342 (1924); ibid., 38, 127 (1926); Kolloidchem. Beihefte, 20, 288 (1924); Biochem. Z., 170, 126 (1926); ibid., 1927); Lowy, "Festschrift" (in preparation).
[2] Halliburton, W. D., J. P. Phil., 5, 155; 8, 133 (1887).
[3] Cohnheim, O., "Chemie der Eiweisskörper," Braunschweig, 1911.

from an electrolyte-free solution, still there are to be found in the literature a great many statements denying the heat-coagulability of completely purified albumin solutions. This view has recently been upheld, particularly by Lepeschkin.[4] Finally, the influence of the H-ion concentration for the coagulation of albumin must be considered.

Since in the production of pure albumin solutions, dialysis is always used--either for the removal of salts present in the original medium or for the separation of the salt used for precipitation--the statements of Schmidt[5] and his pupils Aronstein,[6] Heynsius,[7] W. Kieseritzky,[8] and Al. Rosenberg,[9] who showed that dialyzed albumin can no longer be coagulated by heat, practically mean that salt-free solutions of albumin do not coagulate upon being heated. This latter statement occurs, for instance, in the text-book of O. Hammarsten (1922).

Now Pauli[10] has shown that thoroughly dialyzed serum coagulates between 50° and 60° C., even when freed of globulin by precipitation with half-saturated ammonium sulfate. The above illustrations[11] are intended only to show that, as a result of differing migration speeds of anions and cations, there is established an alkaline reaction at which the proteins are not coagulated by heat, and which comes at a definite time during each dialysis of salt-containing protein. The findings of the above authors and analogous findings of Michaelis and Mostynski,[12] who were able to demonstrate coagulation only near the boiling-point, are explained by early interruption of the dialysis at the time of this alkaline reaction. This explanation of Pauli's is accepted by Sörensen and Jürgensen,[13] as well as by others.

In the above-mentioned investigation, it was necessary entirely to free the horse-serum and the egg-white used in the preparation of albumin from salt and globulin. Bearing in mind earlier observations, the initial dialysis in a folded dialyzer was combined with electrodialysis, thus diminishing the time of dialysis, which many authors consider a possible source of error. The methods employed, and the properties of the soluble proteins obtained by these methods, have been discussed in an earlier work.[14] The conductivity, hydrogen ion concentration, heat-coagulation temperature, and ash content of such albumin solutions are given in Table I.

Most of the investigations on heat coagulation, however (as for instance, the intensive studies of Chick and Martin[15]), were made on material containing ammonium sulfate. In addition, according to these workers, their egg-albumin contained free acid. Consequently, quite considerable quantities of alkali were necessary in order to attain the hydrogen ion concentration of distilled water. Lepeschkin also worked in the presence of ammonium sulfate, while Sörensen,[16] in the course of a description of a study on acid flocculation in his latest communication, states that the egg-albumin solution being studied must be freed of salts as completely as possible. Experimental results

[4] Lepeschkin, N., *Kolloid-Z.*, **31**, 342 (1922); **32**, 42, 44, 106 (1923).
[5] Schmidt, Al., *Pflugers Arch.*, **8**, 75 (1874).
[6] Aronstein, B., *Pflugers Arch*, **8**, 75 (1874).
[7] Heynsius, E., *Pflugers Arch*, **9**, 514 (1874).
[8] Kieseritzky, W., "Die Gerinnung U. S. W.," Diss., Dorpat (1882)
[9] Rosenberg, Al., "Vergl. Unt betr. d. Alkalialbuminate, etz," Diss., Dorpat (1883).
[10] Pauli, Wo., *Beitr. chem. Physiol*, **10** 53 (1907).
[11] Cf. Pauli, Wo. and Wagner, R., *Biochem Z.*, **27**, 296 (1910).
[12] Michaelis, L. and Mostynski, B., *Biochem. Z.*, **24**, 79 (1910).
[13] Sorensen, S. P. L. and Jurgensen, E., *Biochem. Z.*, **31**, 397 (1911).
[14] Adolf, M. and Pauli, Wo., *Biochem Z*, **152**, 360 (1924).
[15] Chick, H., and Martin, C. L., *J. Physiol*, **40**, 404 (1910); **43**, 1 (1911); **45**, 61, 261 (1912).
[16] Sorensen, M. and S. P. L., *Compt. rend. Lab. Carlsberg*, **15**, 1 (1925).

obtained without consideration of this principle would be comparable with the above results only after careful proof that the electrolytes present neither affect properties of the resulting coagulum nor enter into chemical combination with it. In this connection see the initial references given.

In view of the fact that Michaelis, Rona,[17] Mostynski,[12] H. Davidson,[18] etc., have emphasized the significance of the H-ion concentration for coagulating proteins, the reaction of the protein solutions here employed must be discussed. Most of these workers agree in stating that proteins are coagulated only at an acid reaction. The statement was formulated by Michaelis and his coworkers that heat-denatured albumin in an electrolyte-free solution will coagulate only after buffering to the isoelectric point. After Pauli and Wagner (*vide supra*) had explained the observations of Michaelis and Mostynski as being caused essentially by the use of an alkaline-reacting albumin solution, Sörensen and Jurgensen carefully studied the question of the influence of the H-ion concentration on the coagulation of egg-albumin. In agreement with Pauli and Wagner, they explain the phenomenon which was established both by them, and later by Chick and Martin, as follows: Relatively large quantities of HCl are necessary to bring about the heat-precipitation of egg-albumin, in order to neutralize the alkaline salts present. The acid reaction of the protein solutions, which appears to be most favorable for the coagulation of a pure solution, is determined principally by the acid reaction of the albumin itself, when in solution. The decrease in H-ion concentration observed by Chick and Martin during the coagulation, is ascribed to the diminution in amount of soluble proteins thereby occasioned.

After it became possible to demonstrate, in an investigation with W. Pauli,[14] that egg- and serum-albumin purified by electrodialysis show but a slight H-ion concentration, such albumin was heated without the addition of any other substance. Water-insoluble globulin, suspended in distilled water, was treated in the same way, after it had been freed, by careful washing and electrodialysis, from the last traces of any soluble albuminous material and electrolytes contained as impurity.

The temperature chosen, that of boiling water, had already been used by many others, including Sörensen and Jürgensen; for they were concerned, not with the course of the heat-denaturization, but rather with the most complete possible denaturization of the albumin, because of its physical and biological significance and ready reproducibility. These authors were able to determine by means of progressive nitrogen determinations on the filtrate, the optimum time of heating to produce complete precipitation of the albumin, without inducing protein decomposition by the boiling water. They boiled 15 minutes in all cases. According to the investigations of Sörensen and Jürgensen, however, this time is not long enough for the complete coagulation of egg-albumin, i.e., for the attainment of constancy of nitrogen content. Still, these investigators have shown that, in the case of egg-albumin, secondary processes of a hydrolytic nature parallel the course of the heat-coagulation. Consequently egg-albumin was not boiled for long periods. The phenomenon reported by Michaelis and Rona [19] (*vide supra*) that there occurs at first a reversible and then an irreversible denaturization, depending upon the length of time of heating, becomes understandable, because on continuing

[17] Michaelis, L. and Rona, P., *Biochem. Z.*, **27**, 38 (1910).
[18] Michaelis, L. and Davidson, H., *Biochem. Z.*, **30**, 142 (1910).
[19] Michaelis, L. and Rona, P., *Biochem. Z.*, **29**, 294 (1910).

the boiling the reversible is continuously changed over to the irreversible form. To present the formation of too compact a coagulum, and to assure an equalized temperature action on the entire protein, there were used horse-serum albumin solutions of relatively low concentration, i.e., 0.4 per cent. And since, according to Lepeschkin, egg-albumin does not coagulate in very dilute solutions, the heat-coagulation tests were carried out on egg-albumin solutions of 2.4 per cent, 1.2 per cent, 0.6 per cent, 0.3 per cent and 0.15 per cent. During the entire experiment, the liquid was stirred vigorously. In no case was there any evidence of evolution of H_2S during heating. Globulin suspension shows no visible change in its properties after being heated. The dense turbidity appearing in water-soluble proteins under the above conditions formed precipitates which were macroscopically visible in all cases. The filtrates obtained on cooling (with the exception of the most dilute egg-albumin solution, which showed a slight opalescence) were always clear as water, and gave no evidence of turbidity either upon the addition of sulfosalicylic acid, or upon further heating. The biuret reaction, however, was negative only in the filtrate of the boiled serum-albumin. This naturally does not preclude the possibility, upon perfection of the technic, that by application of the biuret reaction to the highly concentrated filtrate, or by application of specific immunity reactions (e.g., the precipitin method), there may be demonstrated traces of albumin which were not coagulated by heating. This may also be the case in Zinsser's [20] experiments.

For our purpose, the degree of coagulation of the serum-albumin attained, may be considered complete. On the other hand, the clear filtrate from the heat-precipitated egg-albumin solution, still showed a significant biuret reaction. This finding is in agreement with the work of Sörensen and Jürgensen, in which they were able to show considerable differences in the heat-coagulation of serum- and egg-albumin. These workers, however, refer to this point only for the purpose of marking the rapidity of the heat-changes within the protein molecule.

TABLE I.

		$K_{uncorrected}$	C_H	Ash Content
0.4 per cent horse-serum-albumin	Before heating	5.46×10^{-6}	5.30×10^{-8}	0.04%
	After heating	7.33×10^{-6}	$\begin{cases}2.84 \times 10^{-7} \\ 2.71 \times 10^{-7}\end{cases}$	....
2.4 per cent egg-albumin.	Before heating	1.097×10^{-5}	1.70×10^{-5}	....
	After heating	1.85×10^{-5}	$\begin{cases}2.93 \times 10^{-6} \\ 3.57 \times 10^{-6}\end{cases}$	....

In the filtrates obtained after the heat-coagulation of the protein, the conductivity and the C_H were both determined (cf. Table I). On comparing these data with those of the genuine albumin solutions, the following facts become evident:

The conductivity of the filtrate, when compared with that of the true protein solutions, seems to be increased in both cases. The opposite might be expected to occur, if the conductivity of the original solution was due solely to the protein and the protein was partially or completely precipitated

[20] Zinsser, H., *J. Immunology*, **9**, 227 (1924).

on coagulation, without the occurrence of further changes. There results, however, a quantitative difference between increase in conductivity of the heat-precipitated serum and that of the corresponding egg-albumin solution.

Without making assumptions as to the origin of the increases in conductivity (for these increases may, as Lepeschkin thinks, be referable to a liberation of originally adsorbed salts), we may nevertheless make the following assertion: the quantity of these adsorbed salts in the case of seralbumin cannot exceed 2×10^{-5}. On the other hand, in the case of egg-albumin, the greater increase in conductivity might be linked with the biuret reaction of the filtrate and the secondary hydrolytic changes of the heated solutions assumed by Sörensen and Jürgensen. The results of the C_H measurements appear to point in this direction.

In agreement with Sörensen's view on the origin of the C_H optimum for heat-coagulation, and with my own observations on serum-albumin already referred to, upon heat coagulation detectible H-ions disappear almost entirely from its solutions. While in the case of egg-albumin, the C_H is decreased, there are always free H-ions present in perceptible amount. Measurements of these values are also in close agreement with the corresponding data of Sörensen. As in the case of the water-soluble proteins, globulin suspensions also show a rise in conductivity after heating, although the initial value equaled that of distilled water even after shaking a 10 per cent dispersion for 24 hours.

To avoid contamination of the precipitate by soluble impurities, it was washed on the filter with distilled water until the washings showed no further increase in conductivity. It was then dried in warm air on paraffined flat glass dishes, and finally pulverized in a mortar. In order to eliminate possible changes in the protein occurring during this treatment, check tests were carried out in all cases with undried material. It was impossible to demonstrate any differences. Since subsequent examination of the heat-precipitated proteins showed great differences among themselves, the results for the various proteins will be discussed individually. To be methodical, the results obtained with globulin will be discussed first.

II

In order to learn the properties of the heat-denatured globulin, and to see from a comparison of these properties with those of the original material the nature of the changes established, the solubility of the heat-changed globulin (H) (present in excess) in alkalis, acids and neutral salts was next determined. If the solubility of the original globulin in these solutions be set equal to 1, the values for the denatured form are approximately 1/5, 1/40 and 1/100 for alkalis, acids, and neutral salts respectively.

Thus the solubility of heat-changed globulin is perceptibly less than that of true globulin, though the extent of this decrease varies with the solvent. Further, by means of conductivity and H-ion activity determinations, as well as measurements of the migration velocities and qualitative transference studies, we were able to show that heat-changed globulin binds both acids and alkalis in a different manner than the original globulin. The changes induced by the action of heat persist even in the presence of these added substances.

Taking into account the assumptions of Hardy, Robertson, and Pauli, the terminal amino and carboxyl groups as well as the peptid linkages suggest

themselves as points of attachment of acids and bases to proteins, which latter are considered for this purpose as amino-acid chains. From the physico-chemical investigation of heat-denatured globulin, it appears that the terminal positions of union have been made unavailable, while there is no reason to believe there has been any change in the position of the peptid linkage.

In the case of heat-precipitated serum albumin, the order of activity of the solvents was substantially the same as with globulin. Solubility in neutral salts is significant. However, closer study of the alkaline solution produced with excess of protein, develops the surprising fact that no precipitation occurs on neutralization; that, therefore, the salt concentration, very low in this case, suffices to hold in solution a relatively large quantity of protein. Heat-precipitated seralbumin, therefore, has changed its properties under the influence of NaOH. This fact becomes quite clear from the following experimental results. If the same alkaline solution of heat-precipitated seralbumin be submitted to electrodialysis, the alkali is quantitatively removed. Nevertheless, all the seralbumin, which had originally been made insoluble through the action of heat, now remains in solution. There is no difference apparent between the latter and unchanged albumin solution. Thus, the heat-precipitated seralbumin has again become water-soluble under the influence of the alkali. For we satisfied ourselves that the change could not be attributed either to electrodialytic action on a heat-coagulated product, or to a possible bacterial or fermentative change.

Now, before interpreting the results of the above investigations, experiments must first be considered which demonstrate influence exerted upon the properties of the dissolved products by varying the proportions between NaOH and heat-denatured albumin. Earlier experiments [21] dealing with globulin III, led to quite a different result, in that heat-denatured albumin, dissolved in the NaOH, precipitated out on neutralization in the presence of salts. The difference was that in the latter case no excess of protein was present.

TABLE II.

No.	Gms. Protein in 10⁻³ Equivalents of NaOH	Dry Weight of the Solution	Sol. Protein After Electro-dialysis	Sol. Protein in per Cent of Dry Weight of the Original Solution	Solubility of the Precipit-able Part in Neutral Salt Solution (0.1 N KCl)
I	4	1.47%	1.47%	100%	...
II	2	1.12	0.80	72	+
III	1	1.00	0.42	40	+
IV	0.5	0.465	..	..	—
V	1 gm. protein in 1×10⁻³ HCl	0.64	0.23	37	+
VI	Residue from No. 1 dissolved in NaOH, so that albumin- and alkali-content correspond with II.	0.76	0.54	71	+

[21] Cf. Adolf, M., Kolloidchem. Beihefte, 18, '70 (1923)

The experiments were carried out thus: Varying quantities of the same heat-denatured albumin were dissolved in 100 cc. of 0.01 *N* NaOH in exactly the same way. (See Table II.) It then appeared that the absolute content of protein of the resulting solutions decreased with the decreasing quantity of the proteins employed, while a constantly decreasing residue of protein remained undissolved. If these liquids were electrodialyzed, the quantity of protein remaining in solution was found to decrease with a decrease in the initial quantity of heat-denatured albumin used, and finally to disappear. That is, from the solution of 0.5 gram of heat-denatured albumin in 100 cc. of 0.01 *N* NaOH, there results a protein solution which is quantitatively precipitated on electrodialysis. There exist, therefore, two limiting cases, within which there are continuous transitions. With a sufficiently large excess of protein, we get only water-soluble protein; with a correspondingly large excess of alkali, only water-insoluble protein. In the first case, it was necessary to determine whether the undissolved excess of heat-denatured albumin had suffered a change, or whether the alkali employed merely dissolved part of it. Now if we mix the same proportions of denatured albumin and alkali, using for our protein the undissolved portion from experiment I, separated as completely as possible from the soluble portion, we find that in the solution thus formed, the water-soluble and the total protein content check with the results of experiment II, within experimental error. This result indicates that the undissolved residues in experiment I had suffered no perceptible change through the first addition of alkali.

Only a single experiment was performed to determine the solubility of heat-denatured albumin in acids and the nature of the resulting solution. This experiment shows that apparently the difference in behavior of acids and alkalis with respect to decreased solubility is only a quantitative one, while the composition of such a solution of soluble and "insoluble" protein approaches fairly closely that of the corresponding alkaline solution. [Experiments in which NH_3 was substituted for NaOH lead to results in quantitative agreement with the above, in that the protein which dissolved when there was an excess of protein, was found to be water-soluble after electrodialysis. However, because of the lowered "dissolving power" of ammonia, these experiments were not continued.] Finally it became evident (see Table II) that the salt-solubility of that part of the protein precipitable by electrodialysis, shows a change with the shifting of the ratio of heat-changed albumin to alkali, the solubility diminishing with decrease of alkali. If all the protein be precipitated by electrodialysis, its salt-solubility is also nil.

Let us attempt to examine more closely the experimental results here given. The first question that arises is: what is the nature of the soluble protein (which we will call here protein X)? And is it, as a product of denaturization, reconverted by the alkali treatment, into something identical with the original albumin? If it be different, we then have an hitherto unknown water-soluble protein.

From the physico-chemical evidence, the product in question is not distinguishable from true albumin. The liquid is entirely clear, and yellow like the original albumin. On half-saturation with $(NH_4)_2SO_4$, it behaves like a typical albumin, whereas primary albumoses which might have resulted from hydrolysis of the heat-precipitated product by HCl and NaOH, are completely precipitated by half-saturation with $(NH_4)_2SO_4$. The reaction of the liquid is substantially that of the original albumin, i.e., weakly acid (cf. Table III).

<div align="center">TABLE III.</div>

	K uncorrected	C_H	$[\alpha]_6^{20}$	Heat Coagu- lation Point for 0.4 Per Cent Solution	Heat Coagu- lation Temperature
1 per cent albumin in question (Protein X)	8.26×10^{-6}	5.3×10^{-6}	66.5	48°	39°
1 per cent genuine ser- albumin	7.8×10^{-6}	5.7×10^{-6}	66.4	48°	...

The C_H determinations are in harmony with the conductivity measurements. The low value of the latter, which equal the conductivity of the original albumin, makes it appear evident (as does the faintly acid reaction) that the alkali used to dissolve the heat-precipitated albumin was almost entirely removed. Protein X also shows complete coagulation on heating. Besides, both proteins show the same coagulation temperature at the same concentration and under the same experimental conditions.

Examination of the optical rotation of protein X led to the results reported in Table III, and after reduction to the same protein content, the values showed a very satisfactory agreement with the published data of Pauli, Frisch and Válkó,[22] for a globulin-free albumin prepared by the same procedure. Therefore it seems to follow from these observations that those groups in the protein molecule whose modification would produce a change in the optical rotation, remained unaffected in spite of the treatment used in the preparation of protein X. The differences between our protein and a typical alkali albuminate which, according to Hoppe-Seyler, shows an increase in its original $[\alpha]_D$ of -60.01 to -86, are quite evident.

Furthermore, we tested the behavior of protein X toward alcohol, gold sol and mastic sol. Qualitatively, it agreed exactly with the original protein. Quantitatively, however, there were small differences, which are also found in the literature with identical proteins having different past histories. Attempts to determine differences between true and denatured albumin by chemico-analytical methods do not appear to be very promising, because of: (1) the negative result of the sulfid-sulfur test; (2) the trivial rise in conductivity after coagulation; and (3) the last work of Sörensen initially referred to—from which it appears that the heat-coagulation of egg-albumin, like alcohol-precipitation, takes place without the evolution of nitrogen.

In order, however, to try a further criterion for the characterization of protein X, the highly sensitive immuno-biological methods were employed. For the purpose of identifying proteins, practical examples of the application of the precipitin method,[23] of anaphylactic reactions,[24] and of complement-fixation tests[25] were readily found in the literature of serology. For reasons explained more fully below, the precipitin method was selected.

After R. Kraus[26] had observed, following bacterial inoculation, the pres-

[22] Pauli, Wo., Frisch and Válkó. *Biochem. Z.*, 164, 401 (1925).
[23] Obermayer and Pick, E. P., *Wien. Klin. Rundsch.*, No. 15 (1902); *Wien klin. Wochensch.*, p. 659 (1903); No. 12 (1906). Schmidt, W. A., *Biochem. Z.*, 14, 294 (1908) Bibliography.
[24] Pick, E. P., and Yamanouchi, *Wien klin. Wochenschr.*, No. 44 (1908); *Z. Immunitätsforsch.*, 1, 676 (1909); Doerr, R. and Russ. *Z. Immunitätsforsch.*, 3, H. 7 (1909); Wells, *J. Infect. Diseases*, 5. No. 4 (1908); Besredka, *Ann. l'Institut Pasteur*, 21, 950 (1907).
[25] Wells and Lewis, *Proc. Am. Soc. Biol. Chemists*, 18th meeting, Dec. 27 (1923).
[26] Kraus, R., *Wien. klin. Wochenschr.*, No. 32 (1897).

ence of substances in the sera of animals which gave specific precipitations with cell-free filtrates of cultures of the homologous organism, Bordet [27] and Tstistowitsch [28] contrasted the substances thrown down as protein precipitins (after inoculation with protein compounds) with the so-called bacterial precipitins. It then developed that these protein precipitins are highly specific; i.e., they react only with the proteins originally used. Now while the species specificity of proteins has to a large extent been studied only for forensic purposes, Obermayer and Pick showed that both chemical and physicochemical disturbances of the protein molecule are capable of preventing its visible reaction with native immune serum. However, these disturbances change the antigenic character of the proteins to such an extent, that immune sera produced by them react with the changed proteins markedly if not exclusively.

The experimental results are given in Table IV.

TABLE IV. *0.1 cc. Precipitin to 1 cc. Albumin-NaCl-Mixture.*

Dilutions with 0.85 Per Cent NaCl	Per Cent Horse Serumalbumin	Preparation I (cf. Table II) After Electrodialysis	Dog Serumalbumin	NaCl	Preparation IV After Electrodialysis	Albumin + NaOH to a Final Concentration of 0.005N	Albumin + NaOH to a Final Concentration of 0.005N and Boiled	Horse Coctoserum
1 : 100	+++	+++	0	0	+	+++	0	—
1 : 1,000	+++	+++	0	..	+	+++	0	—
1 : 10,000	++	++	..	..	+	++	0	—
1 : 100,000	+	+	..	..	0	+	..	—
1 : 1,000,000	0	0	..	..	..	0	..	—

They indicate that electrodialyzed horse-serum-albumin is carried down by the precipitating horse-serum even on extreme (1 : 100,000) dilution. The negative result of the control test demonstrates the specificity of the reaction. As is apparent from the table, protein X shows the same behavior in the precipitation test as the original albumin. It was impossible to demonstrate the characteristic failure of coctoserum to react with the normal serum precipitin of the proteins here investigated (as described by the authors initially referred to, and which my own researches confirmed—cf. Table IV), although the former was kept at a temperature of 100° for 15 minutes.

In two other series of experiments we tested both the antigenic properties of protein X and its relation to a precipitating serum prepared with a heated serum as antigen. In both cases protein X fails to show the characteristic behavior of heat-changed albumins, and here too behaved just like the original albumin.

Accordingly, no essential difference is demonstrable between protein X and its parent material (a true serum albumin) by the physico-chemical and immuno-biological methods here employed. The agreement is so close that,

[27] Bordet, *Ann. Institut Pasteur*, 173 (1899).
[28] Tstistowitsch, *Ann. Institut Pasteur*, 406 (1899).

provided no new experimental results arise to contradict it, we are forced to admit an actual identity of the two proteins. Such an identification, however, would imply that the changes produced by heat had been made chemically reversible by the treatment given the heat coagulated albumin. Indeed, according to Pauli [29] this is a case of *heterodromic reversibility*, since the reversal proceeded to the starting point by a different path.*

The heat denaturization of proteins is generally considered to be irreversible (cf. Pauli), since no general chemical method for reversal is known.** Thus Cohnheim states that coagulated protein cannot be dissolved without extensive splitting and changes in its original properties, and that it remains permanently denatured. The few statements to the contrary, such as that of Corin and Ansiaux,[30] which has since been refuted by Pauli,[29] and that of Michaelis and Rona (*loc. cit.*), are concerned principally with the first stage of coagulation. Thus the latter authors have stated that only such albumin as is heated for a short time and is dissolved in HCl, possesses the property shown by genuine albumin, of coagulating at its isoelectric point; that, however, heating for several minutes is sufficient to change the albumin irreversibly. Let us recall, however, that the albumin here employed had been kept constantly at the boiling point for 15 minutes. Likewise, a paper of Hoppe-Seyler on this subject, which discusses a limitation of the temperature of denaturization, contains a similar statement regarding the duration of the heat treatment.

Besides the chemical points of view just presented concerning the possibility of a generally attainable reversal of the heat denaturization, it is interesting to note that immuno-biological experiments have led to practically the same conclusion (cf. Schmidt, and especially Wells and Lewis). Summarizing the discoveries made with heat-changed globulin and seralbumin, we may conclude that it is principally the terminal groups of the protein molecule which, under suitable experimental conditions, undergo reversible changes.

Let us now attempt to use the above established facts concerning the behavior of denatured globulin and seralbumin in deciding what changes heat produces in the protein molecule. We must again emphasize that physicochemical analysis gives neither direct nor unequivocal evidence of the existence of a definite chemical change. Still most of the authors treating this phenomenon have paid no attention to the numerous explanations possible, and have postponed the problem of heat denaturization until the alternative question has been settled—whether the process is essentially a ring formation with condensation, or an hydrolysis.

If we wish to use the reversibility produced by alkali treatment of the heat-coagulum to interpret the changes in the protein molecule which govern this reversability, it is first necessary to prove that this phenomenon is characteristic of the heat-coagulum. Accordingly, the behavior of protein coagula which had been produced by other reactions, was tested to the same end. Alcohol,[31] ultraviolet light (Spiegel-Adolf: *loc. cit.*) and radium rays [32] were

[29] Pauli, Wo., "Kolloidchemie der Eiweiskoper," Steinkopff, Dresden, 1920.
* It seems quite possible that heat causes an aggregation of the protein molecules (consequent on their close approach) which is not reversible unless a peptizing substance (e.g. acid or alkali) be present. An analogous case is found with gelatin-collogen. See J. Alexander, "Allen's Commercial Organic Analysis," 4th ed., Vol. VIII, p. 586. *J. A.*
** Only very recently, since the appearance of my studies on this subject, Wilhelm has stated that the action of salts with complex ions, on heat-changed proteins, results in similar phenomena which might be considered reversibility.
[30] Corin and Ansiaux, *Bull. de l'Acad. roy. de Belg.*, 21.
[31] Adolf, M., Unpublished data.
[32] Adolf, M. and Fernau. Unpublished data.

chosen as denaturing agents. In a series of experiments which, although not analogous to those above, were chosen as convenient and comparable, it was shown that the water-insoluble seralbumin produced by alcohol denaturization can be transformed into a water-soluble, heat-coagulable substance, by the action of alkali and subsequent removal of electrolytes. Egg-albumin did not show this property; neither did either of the radiation-coagula of seralbumin.

From these experiments it seems that reversibility of the coagulum is not shown by all denaturized products, but is characteristic only of certain of them, and seems well suited to the demonstration of differences regarding which very little appears in the literature. Referring to the question initially propounded, it seems significant that just that product produced by ultraviolet rays, which, according to Neuberg,[13] first causes hydrolysis of the protein, that can not be made reversible. On the other hand, alcohol, acting as a dehydrating agent, forms a coagulum which manifests the same behavior as the heat-coagulum. In spite of the brilliant work of Wu,[14] these results seem valuable only as theories which do not prove heat-denaturization to be a hydrolysis of the protein.

The demonstration that the changes brought about by heat can be reversed by small amounts of alkali or acid, supports the view that ring closure accompanies the heat-change. For, while the action of these added substances, which persists even after their electrodialytic removal, can be interpreted as an hydrolysis, there might possibly have been involved a breaking down of a pre-existing ring structure. On the other hand, it is not obvious how any previously occurring hydrolysis can be completely reversed by an added substance which itself is known to aid hydrolysis. The above findings seem to strengthen the theory that the change appearing upon heat-denaturization of the proteins depends upon a ring closure of the groups involved. It is impossible to determine here, whether the sensitivity shown by heat-precipitated albumin to the denaturing influences of relatively large quantities of alkali (similar experiments with genuine albumin, after electrodialysis, show there has been no change), depends upon the peculiar properties of heat-denatured albumin caused solely by hypothetical ring formation, or is conditioned by other changes which are reversible upon slight hydrolysis.

Finally, we may refer to the fact (connected with the formation of "artificial globulins" from albumin), that on denaturing heat-coagulated albumin with alkali or acid, salt-soluble products can be obtained by the use of relatively small quantities of these agents; whereas the salt-insoluble substance obtained with the increased addition of alkali is considered an alkali-albuminate. Starke[35] and Moll[36] have described the formation of salt-soluble products under certain conditions (i.e., the combined action of alkali and heat), and have classified them as globulins. Without discussing here in greater detail the question of globulin formation, let us consider the phenomenon itself.

The above experiments have confirmed Moll's results with seralbumin in so far as this author considers the action of heat, and of alkali, necessary for the production of the substance he describes. However, they indicate that the action of heat can be distinguished from that of the alkali, and that by

[13] Neuberg, C., *Biochem. Z.*, **13**, 305 (1908); **29**, 279 (1910).
[14] Wu, H., 1927
[35] Starke, Joh., *Z. Biol.*, **40**, 494 (1900)
[36] Moll, L., *Hofm. Beitr.*, **7**, 311 (1905).

acting upon the heat-precipitated product with varying quantities of alkali, the protein passes through all stages from a water-soluble product, indistinguishable from the original material, to a salt-soluble and finally to a salt-insoluble product. Since the quantities of alkali used in the latter case do not affect genuine albumin at room temperature, it is questionable whether the salt-insoluble product resulting from the treatment of heat-changed albumin is to be classified as a true alkali albuminate, or whether it is not much more justifiable to consider the salt-soluble fraction as a distinct protein—as Moll has already done. In this particular case it appears to be a substance intermediate between proteins which are soluble in water, and those which are not. But these facts, which may be supplemented by data drawn from the field of globulin research, permit us simply to conclude that derivatives of heat-coagulated albumin obtained under quantitatively fixed conditions, are salt-soluble just like the globulins.

A necessary prerequisite for the reversibility of the heat change, is that it be not accompanied by secondary decomposition processes of the protein. Such processes, however, are observed upon heating egg albumin. As a result of the explanation of the properties of the seralbumin already given, it should be possible to anticipate several characteristics of the heat-changed product in the case of egg albumin. Suitable experiments clearly demonstrate this to be so. In order to demonstrate analogous behavior in alkali-egg-white, heat-precipitated egg albumin was partially dissolved without previous drying, by shaking for two ten-hour periods. The dry weight of this solution was 1.7 per cent. The solution was turbid, in contrast with the corresponding ser-albumin preparation, and translucent only in thin layers; but it was quite stable. On neutralization, as well as on electrodialysis, the protein was thrown down quantitatively. So that, in contrast with seralbumin, no formation of a water-soluble modification of the heat-precipitated albumin on treatment with alkali, could be observed under like conditions. On the other hand, heat precipitated egg-albumin in alkaline solution shows complete salt-solubility on neutralization in the presence of an exces sof salt. Heat-changed egg-albumin, exposed to the action of alkali in the presence of excess of protein, shows a close agreement with seralbumin treated with an excess of alkali, and also exhibits the typical behavior of globulin solutions under like conditions. Thus saline solutions of heat-changed egg-albumin are precipitated on dilution with water, precisely as are similar solutions of globulin; likewise upon the introduction of CO_2 following addition of HCl in quantities insufficient for resolution. One third saturation with $(NH_4)_2SO_4$ induces extensive precipitation; half saturation throws it down completely.

The agreement in behavior between globulin and heat-precipitated egg-albumin is also shown by the change in H-ion concentration (measured potentiometrically) of salt solutions on increasing dilution. We have already shown that seralbumin, under proper experimental conditions, exhibits a diminution of H-ion concentration; but a 0.1 N KCl solution of heat changed egg-albumin, showed an acidity increase like globulin solutions of comparable salt-content. Table V gives two such dilution series for convenient comparison. In addition, other experiments are recorded in which the protein concentration was maintained constant. Finally from the last series (which is contradicted by a similar one in globulin VII [37]) it follows that the phenomenon is not only induced by the relatively high salt concentration, but

[37] Adolf, M., *Kolloidchem. Beitr.*, 21, 241 (1926).

TABLE V.

Dilution with H_2O	0.1N KCl + Heat-changed Egg Albumin	0.1N KCl + Globulin	0.2N KCl + Heat-changed Egg Albumin (Diluted 20 Times with 0.2N KCl)	0.0008N Na-heat-denatured-egg-albumin + KCl (to a Final Salt Concentration of 0.102N)
	$C_H=5.3\times10^{-6}$	$C_H=5.76\times10^{-6}$	$C_H=2.13\times10^{-6}$	$C_H=1.18\times10^{-6}$
1:1	$C_H=7.28\times10^{-6}$	$C_H=7.79\times10^{-6}$	......	$C_H=9.65\times10^{-7}$
1:3	$*C_H=8.88\times10^{-6}$	$*C_H=9.39\times10^{-6}$	......	......
1:7	......	......	......	$C_H=4.92\times10^{-7}$
1:19	......	......	$†C_H=1.17\times10^{-6}$	......

* At the beginning of the precipitation.
† In the clear supernatant liquid.

is dependent upon the exact neutralization of the Na-proteinate to its neutral salt compound.

We then showed that heat-changed egg-albumin in KCl solution behaves exactly like globulin in migration experiments. Like the latter, the heat-changed egg-albumin migrates towards the cathode (cf. Table VI). Therefore it would appear that the behavior of globulin closely resembles that of heat-precipitated egg-albumin, even in its physico-chemical behavior in the presence of neutral salts. The significance of these and similar phenomena will be taken up later.

TABLE VI. *Dry Weight of Protein in All Experiments = 0.85 Per Cent.*

Final Conc. of Salt	Description of the Experiment	Direction of Migration	Remarks
0.2N KCl	Layered over with 0.2N KCl—100V— 60 seconds	K + Λ —	Detected with sulfo-salicylic acid
0.2N KCl	as above—38 seconds	K + Λ —	Detected with sulfo-salicylic acid
0.1N KCl	Layered over with 0.1N KCl—50V— 30 seconds	K + Λ —	Detected with sulfo-salicylic acid

V = volts   K = cathode   Λ = anode
+ = present   — = absent

As has already been pointed out, Starke has shown that heating of hens' egg-white under certain stipulated condition (e.g., very low salt concentration, slightly alkaline reaction, and heating at 56°) leads to the formation of a globulin-like product. Judging from the solubility of this substance, he concludes that it is not a coagulated protein, even though it loses its solubility in acids and alkalis when heated at 95° with neutral salts. We were able to show that a similar phenomenon is observable if the egg albumin is heated with small quantities of $(NH_4)_2SO_4$. In this case, a product is formed which is insoluble in 0.1 N NaOH.

From the above, two facts emerge; first, the similarity in behavior between the solutions of heat-precipitated albumin and the Starke's substances; second, the fact that the presence of neutral salts seems to be significant not only for the aggregation of small particles of denatured protein, but also for the nature of the coagulum produced by the action of heat. All this accords with the results of similar experiments performed with globulin.

III

In order to localize the heat-changes in the protein molecule, recourse was had to the behavior of the protein heated in the presence of acid and alkali. Kestner (Cohnheim) has interpreted prior experimental results on water-soluble material to mean that the denaturization of proteins upon heating occurs in every case, with or without salt, but that the fate of the resulting denatured protein varies, depending upon whether or not salt is present. Experiments with globulin gives conflicting evidence. Solutions prepared in the presence of excess globulin in .01 N HCl and .01 N NaOH, showed no changes in their outward appearance, in H-ion activity, or in conductivity, even after being heated to the boiling point for 15 minutes. The power of combining further with acid or alkali, in the presence of excess quantities of these substances, remained unchanged. An appropriate series of experiments showed that no change had occurred in the power of combining with alkali in the case of heated acid-globulin, and with acid in the case of heated alkali-globulin. Finally, it was possible to point out that the neutral salt solubility of the globulin contained in these solutions had remained unchanged, in spite of the heat to which they had been subjected.

The above experimental results, then, give no basis for believing there is a denaturization of protein following heating of acid- or alkali-globulin. On the contrary, the experiments show that the product is entirely different from the one obtained by heating in suspended form, whose original characteristics are completely altered; but that in physico-chemical tests it does not differ from genuine globulin.

It is possible only to guess at the mechanism which underlies inhibition of heat-changes consequent on the presence of acid or alkali. Since salt-globulin is completely precipitated on heating, then, in addition to the electric charge of the protein ions, the ionic combinations of terminal groups must also be of significance. The discrepancy between these findings and the results with albumin mentioned above, made a re-examination of this last protein substance urgently desirable.

Since it is known that different products result from the reaction of albumin with acids and alkalis under like conditions, the behavior of albumin upon heating at an acid or an alkaline reaction will be next discussed. First we will discuss experiments with seralbumin. In all cases, the albumin with the respective electrolytes was heated on a wire gauze for ten minutes to the boiling point. To indicate any change which might have taken place in the proteins, the water-solubility was tested, after electrodialytic separation of the electrolyte initially added. After it had been shown in control experiments that genuine albumin remains unchanged when so treated, this method appeared to be superior to neutralization and bringing to the isoelectric state, as employed by Michaelis. For this author himself indicates the possible influence of neutral salts on the results obtained by his procedure, and, what is more, this

view is supported by the above-mentioned indication of the formation of salt-soluble protein upon treatment of the heat-coagulated albumin with alkali.

We next determined the quantity of HCl capable of preventing all visible change in the protein on heating, when the albumin content is constant. In the actual experiments, it was necessary to add to this minimum quantity of acid an amount which previous experiments showed to be just sufficient to bind the protein present. This was done in order to avoid any possible excess of protein, as well as to carry out the experiments under conditions comparable with those on globulin salts. However, in order to discover any possible influence of the bound acid as well as of free H-ions, the acid concentration was varied. In addition to HCl, H_2SO_4 and acetic acid were also employed in order to increase the generality of the data obtained (cf. globulin II). From these results, summarized in Table VII, it appears that in none of the cases investigated was the albumni completely transformed into a water-insoluble modification on boiling in the presence of acid. In fact the amount of the water-insoluble product decreases with increasing acid concentration, only to increase again upon further addition of the reagent. In the determination of the minimum amount of water-insoluble protein present, it was found that more than 90 per cent of the initial material is water-soluble and heat-coagulable.[38] Experiments with H_2SO_4 and acetic acid give similar results, thus demonstrating that the partial or entire absence of heat changes in solutions of acid-protein is almost completely independent of the degree of hydration of the latter. On neutralizing and cooling the heated acid solution, an appreciable cloudiness appears in all cases except that of the highest acid concentration, but only after several hours. It may be that the quantity of salt formed during neutralization is sufficient to keep the water-insoluble product in solution. This is all the more plausible since the concentration of protein employed is a low one. Indeed, Michaelis and Rona have already expressed this view, and besides this explanation is supported by the fact that the addition of neutral salts—at least in low acid concentrations—may prevent precipitation upon neutralization. These results show, on the one hand, a certain agreement with the observations of Michaelis and Rona according to which albumin, heated in the presence of larger quantities of acid, gives no precipitate on neutralization. On the other hand, they agree with experiments dealing with the production of acid-albumin.[39] In a previous communication it was reported that, by using HCl of such strength as to give a final concentration of 0.04 N and a protein content of more than 1 per cent, after heating, neutralizing and removing the precipitate by filtration, the process could be repeated two or three times by successive addition of proper amounts of HCl—until all the soluble protein was changed to acid-albumin.

In view of these findings it would seem preferable to employ this method of precipitation of the acid-albumin by means of electrodialysis, rather than the usual neutralization procedure. However, this agreement between the above results (obtained with purest albumin) and the earlier ones (in which thoroughly dialyzed beef-serum, kept for years, was used) offers a certain guarantee that the phenomenon of acid-albumin formation is not simulated solely because of globulin residues in the solution.

Let us now consider the corresponding results for globulin, which appar-

[38] After this investigation had been completed a paper by Hsien Wu and D Y Wu was found, in which it is stated that 0.5 per cent horse-serum is not denatured on being heated with 0.05 N HCl, because it can be reconverted to a heat-coagulable form
[39] Adolf, M. and Spiegel, E., *Biochem. Z*, **104** 175 (1920).

TABLE VII. *Initial Concentration of Albumin—0.53 Per Cent.*

	0.0013N		0.0026N		0.005N		0.01N		0.02N		0.04N	
	S.A.*	Per Cent of Initial	S.A.	Per Cent of Initial	S.A.	Per Cent of Initial	S.A.	Per Cent of Initial	S.A.	Per Cent of Initial	S.A.	Per Cent of Initial
HCl	0.15	28	0.44	83	0.49	92	0.4	75	...	...	0.3	56
H_2SO_4	...	...	...	...	0.40	77	...	...	0.4	75	...	...
CH_3cOOH	...	...	...	...	...	...	0.4	75	...	...	...	...

* Soluble albumin.

ently show a certain agreement with those for albumin. In a reaction between albumin and HCl, 90 per cent of the protein shows no demonstrable change on being heated. (According to Manabe and Matula,[40] this reaction corresponds to the formation of a neutral salt, in that practically all the HCl appears to be bound. It is, however, impossible to believe there is any excess of protein here, since further addition of HCl leads to an increase in the H-ion concentration.) The increase in amount of water-insoluble product occurring on denaturization in the presence of both higher and lower concentrations of acid, does not contradict the observations on globulin, since the latter were obtained under different conditions, among them being the use of water-insoluble protein. Thus it is possible to account for the formation of larger quantities of insoluble proteins when lower acid concentrations are used, by the fact that not all the terminal NH_2-groups are bound by acid, and are therefore susceptible to the influence of heat. On the other hand, water-insoluble globulin can be affected by heat only in the form of an acid salt. The increase in amount of the water-insoluble portion by the use of higher acid concentrations, which is not the case with globulin, must be considered a consequence of the effect of a high temperature upon the albumin-salt in the presence of excess acid.

Accordingly, there is no reason why we should not apply, *ceteris paribus,* to globulin, the explanation given for the effect of heat on neutral globulin chloride, and accept the view that groups in the albumin molecule, formed by ionization, exert an acid binding power which not only inhibits any visible change, but also prevents any change at all upon heating.

TABLE VIII. *Final Concentration of Albumin—0.53 Per Cent.*

Final concentration of NaOH	0	0.001N	0.002N	0.003N	0.005N	0.02N	0.05N
Behavior on boiling	XXX	++	0	0	0	0	0
Heated and electro-dialyzed	....	....	0.36 per cent water-soluble	> 0.01 per cent water-soluble	Complete precipitation		

40 Manabe, K. and Matula, J., *Biochem. Z.,* 52, 369 (1913).

These results show that it is erroneous to assume that a heat denaturization of the protein occurs in every case. Further, the phenomena here observed—according to which the intensified ionic character of the terminal protein groups is sufficient to inhibit the occurrence of heat changes—are in accord with the theory that the heat changes consist in ring formation involving these groups.*

If now, we heat albumin in the presence of NaOH, under experimental conditions exactly comparable with the above, we find a close agreement with the experiments on seralbumin, in that with definite proportions of alkali and albumin, the albumin is, in part at least, not denatured on heating. While, however, in the case of acid-albumin there is a relatively wide range of acid concentration (extending from about 0.001 *N* to above 0.04 *N*) within which water-soluble albumin can still be obtained after heating, the corresponding zone for alkali-albumin is considerably narrower. For the same protein concentration, 0.001 *N* NaOH is not able completely to prevent change in the albumin on boiling; while, at a final concentration of 0.003 *N,* the greater part of the albumin becomes insoluble in water. At a concentration of alkali exactly equivalent to the quantity of protein present, the albumin undergoes such changes on being heated that it is completely precipitated by the subsequent electrodialysis. Increasing the amount of alkali employed has no effect in this behavior (cf. Table VIII).

The narrowness of the alkali concentration zone in which denaturization of the albumin is partially prevented explains, for instance, the statement of Michaelis and Rona, that the addition only of acid and not of alkali can result in a water-soluble product, after subsequent neutralization under certain conditions. As to the fact that albumin already gives evidence of becoming water-insoluble at concentrations of alkali so low that it is practically entirely bound by the protein, we might remark that, in part from experiments on the methylation of proteins, Pauli (*loc. cit.*) accepts the peptid linkage as well as the free carboxyl group as loci of alkali fixation. The above experimental results would then warrant the view that in the case of alkali globulin also, the ionic combination of terminal groups can inhibit the effect of heat. With further addition of alkali, in spite of its extensive fixation by the albumin, the latter becomes insoluble upon heating. These considerations have been given additional support by further experimentation.

Studies carried out to this end were based upon the following considerations: If the fact that the albumin becomes insoluble after being heated with alkali, indicates that ionic compounds involving the terminal carboxyl groups are unable to prevent the heat transformation, then the product formed in this way must be identical with that formed on the treatment of heat-coagulated albumin with NaOH under conditions which are exactly comparable quantitatively.

* Heat, alcohol, etc., which partially remove protective water films about protein molecules, may permit the molecules to approach so close that their residual valence forces are made much more highly effective. A definite ring formation, in the chemical sense, does not seem essential, though not impossible. The potent effects of traces of water were brought out by the striking experiments of H. B. Baker [*J. Chem. Soc.*, 121, 568 (1922)], who showed the following rises in boiling points after drastic drying over P_2O_5:

Substance	Years Standing	Rise in Boiling Point	Substance	Years Standing	Rise in Boiling Point
Bromine	8	55 deg.C.	Carbon tetrachloride	9	34 deg.C.
Mercury	9	62	Ethyl ether	9	48
Hexane	8½	14	Ethyl alcohol	9	60
Benzene	8½	26	Methyl alcohol	9	54
Carbon disulfide	1	30	Propyl alcohol	9	39

If the removal of a trace of water can raise the boiling point of ethyl alcohol from about 78°C. to about 138°C., it is not surprising that a protein is greatly influenced in its behavior by anything affecting its water-sheath. *J. A.*

The determining experiment—heating albumin to the boiling point with the addition of alkali, cooling, neutralizing with HCl, filtering off the precipitate, washing and dissolving the latter in NaOH—showed that the product formed in this way was precipitated quantitatively on subsequent electrodialysis, while the solution in alkali of an equivalent quantity of heat-coagulated albumin contained up to 72 per cent of the water-soluble protein. We must assume, therefore, that on heating albumin with alkali, even though free hydroxyl ions be absent, there occurs a change different from the simple heat-denaturization—perhaps the formation of an alkali albuminate may be involved. Up to a certain point, the experiments with alkali-albumin are comparable with corresponding experiments with acid-albumin and with globulin. However, they do not militate against our concept of the mechanism which we believe may account for the heat-change in proteins.*

On the other hand, analogous experiments with egg-albumin, results of which are tabulated in Table IX, confirm the fact that this substance develops a behavior different from that of seralbumin. After heating and electrodialysis, the entire mass of albumin is found to be water-insoluble, even in such concentrations of acid and alkali as preliminary experiments showed to give low H- and OH-ion concentrations.

TABLE IX. *Final Concentration of Egg-Albumin—1.2 Per Cent.*

Final concentration of HCl	0.0025N	0.005N	0.01N
Behavior on boiling...............	XX	0	0
Heated and electrodialyzed........	....	Complete precipitation	
Final concentration of NaOH......	0.003N	0.004N	0.005N
Behavior on boiling	XX	0	0
Heated and electrodialyzed........	....	Complete precipitation	

For the purpose of comparison, corresponding experiments were also carried out with egg-albumin and the same acid and alkali concentrations. Since in this series of experiments, the egg-albumin remained water-soluble, it does not seem justifiable to refer the preceding experimental results to the electrodialysis. The differences in serum- and egg-albumin (which had already been indicated by the differences in the coagula obtained by heat-precipitation in electrolyte-free medium) also manifest themselves by the behavior of these proteins under the influence of heat in the presence of acid and alkali.

However, it still remains to be shown that egg-albumin heated with acid or alkali differs markedly by reason of its insolubility in salt solutions after neutralization, from albumin which is precipitated by heat in an electrolyte-free medium.

Consequently, since we find there is no change in practically neutral compounds of globulin and seralbumin, when heated with acid or alkali (in spite of complicating reactions in the case of the latter protein), it is necessary to

* It seems quite possible that the presence of alkali may favor the maintenance, at least in part, of protective water films, and thus render the protein more "soluble" or hydrophile. The forces holding these films are presumably residual free fields or secondary valence forces, which can hardly be the same for each protein, and may very well differ in various specimens of the same protein. We should not expect of them the same preciseness we find in true chemical combination; the bigger the molecular group, the more protean their effects. *J. A.*

TABLE X.   *Final Concentration of Egg-Albumin—1 Per Cent; Heated with Acid and Neutralized.*

Final concentration of HCl	0.01N		0.02N		0.03N	
Final concentration of KCl	0	0.1N	0	0.1N	0	0.1N
	XXX	XXX	XXX	XXX	XXX	XXX

TABLE XI.   *Final Concentration of Egg-Albumin—1 Per Cent; Heated with Alkali and Neutralized.*

Final concentration of NaOH	0.004N		0.005N		0.01N	
Final concentration of KCl	0	0.1N	0	0.16N	0	0.33N
	XXX	XXX	XXX	XXX	XXX	XXX

investigate the influence which addition of neutral salt exerts upon the manifestations of the denaturization process. Comparative experiments with egg-albumin were also carried out.

Regarding albumin, the experiments confirm the observation that in every case, on being heated with acid or alkali, the protein undergoes a change. Its precipitation under the influence of acid or alkali is inhibited, but in the presence of neutral salts this inhibition is more or less abolished. Since as a result of the previous experiment, it was expected that the globulin would exhibit a simple behavior, the influence of added salt on the acid- and alkali-globulin was next investigated. In every instance we tested the activity of the added salt, before and after heating the solution. It then became evident that the initiation of precipitation is dependent upon whether the addition takes place before or after the heating of the globulin solution. When salts with mono- or divalent ions are used, precipitation occurs only in case the salt is added before the globulin solution is heated. Addition of salt to the solution after heating and then cooling it, exerts no effect upon its initial behavior.

This phenomenon was interpreted to mean that, in contrast to the simple globulin salts in aqueous solution, the presence of neutral salts effects such changes in the condition of the globulin salt as to produce heat lability. However, there is no reasonable ground for dividing the heat change into denaturization and coagulation.

Corresponding to the complicated reactions of heated acid- and alkali-albumin, neutral salts might also produce several effects; for the proteins contain water soluble albumin as well as acid- or alkali-albumin, in proportions which vary with the concentrations employed. First, we must determine whether the water-soluble albumin does not react differently to neutral salts than does genuine albumin; second, we must ascertain whether the acid- or alkali-albumin retained in solution by acid or alkali, is precipitated solely by the addition of salt or then requires reheating. In order to provide conditions best adapted for observation, concentrations of acid- and of alkali-albumin were chosen, which would yield a maximum amount either of the water-soluble

TABLE XII.—Final Concentration of Albumin in All Experiments—0.53 Per Cent.

Final Concentration of KCl	0.02N		0.04N		0.05N		0.066N		0.075N		0.1N		0.2N		0.3N		0.5N	
	a*	p*	a	p	a	p	a	p	a	p	a	p	a	p	a	p	a	p
0.0024N HCl	+	0	++	0	+++	0	+++	0	XX	0	XXX	0	XXX	0	XXX	0	XXX	0
0.005N HCl	0	0	0	0	0	0	0	0	0	0	0	0	+++	0	XXX	0	XXX	0
0.01N HCl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	0	XXX	0
0.04N HCl	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	XXX	0

TABLE XIII. Final Concentration of Albumin in All Experiments—0.53 Per Cent.

KCl	0.01N		0.25N		0.5N		1.0N	
	a*	p*	a	p	a	p	a	p
0.002N NaOH	0	0	+++	0	XXX	0	XXX	0
0.003N NaOH	0	0	0	0	0	0	0	0

a = ante = added before heating.
p = post = added after heating.

or of the insoluble modification. Since corresponding experiments with salts were available only in the case of KCl [40] and since my own investigations in this direction were beyond the scope of this work, KCl only was used, in contradistinction to the investigations with globulin. The results are given in Tables XII and XIII.

We see that if the salt be added to the heated and cooled solution, precipitation does not occur. These results, on the one hand, are in complete agreement with the assumption made in these investigations, namely: that the albumin which remains water-soluble after heating is identical with the genuine albumin, because it shows no change under the conditions of salt-concentration here employed. On the other hand, these studies show that acid- and alkali-seralbumin behave similarly to globulin with respect to neutral salts. If, however, the KCl is added before the solution is heated, then precipitation occurs under definite experimental conditions, which (according to the observations of Pauli [1] on seralbumin, at a constant protein concentration) are dependent on the salt-acid or salt-alkali ratio. Then the above experimental results may be taken to mean that a precipitation which follows the addition of salt during heating, does not depend on the manifestation of a denaturization already effected, whereby either the albumin was not changed at all, or else the changed product was not precipitated by the addition of salt. The precipitating action resulting from the presence of salt can hardly be solely dependent upon the change in H-ion activity brought about by the presence of salt, since this value must be affected to the same extent whether the addition of salt follows or precedes the heating. For the same reasons, we can not explain the phenomenon as due to the formation of an insoluble compound of the nature of a double salt composed of the salt of the denatured albumin and the neutral salt.[1] The experimental results appear, rather, to point to the fact that, as with globulin, the heat-lability of the albumin salt brought about by the influence of KCl, is necessary for heat-precipitation in acid- and alkali-albumin in the presence of a neutral salt. According to the results here described, it is impossible to speak of a denaturization of the albumin in the sense hitherto attached to it, which would take place in the presence of acid and alkali under all conditions but manifest itself only upon addition of salt. Rather, it appears that, with suitable concentrations of egg-white and of acid or alkali, either changes on heating are entirely absent, or else the resulting products of denaturization are different from those of a pure heat coagulation. Since salt causes precipitation only when added before heating, its activity can be interpreted only as the reaction of a compound modified during the process, and not as the demonstration of a denaturization already established. However, the albumin, changed as it may be shown, is not precipitated by the addition of salt alone, but is coagulated only after a second heating. This latter behavior seems to be characteristic also of the denaturization product of seralbumin.

If we compare the preceding findings with those made on egg-albumin, Tables XIV and XV, we find even in experiments with alkali, a satisfactory agreement with the corresponding experiments on seralbumin. Addition of salt produces precipitation if it is made before the fluid is heated. The effective amount of salt depends on the concentration of alkali. However, even in a concentration of NaOH barely sufficient to prevent a visible change on heating, KCl even up to a concentration of 0.5 *N*, if added after heating and cooling of the liquid, does not produce turbidity. On the other hand, it

quantitatively.[29] These facts, indeed, appear to contradict the assumption as to the chemical basis of the changes induced in globulin and albumin by heat. For here we come in contact with proteins whose similar chemical composition and physico-chemical properties would indicate similar behavior, but which nevertheless fail to show visible changes on heating. However, we must remember that, according to the views of Greenberg and Schmidt,[43] the free carboxyl groups of glutamic, aspartic, and β-hydroxy-glutamic acids and the hydroxyl groups of tyrosin must be considered, as positions in the protein molecule where alkali is bound; furthermore, that our knowledge regarding the amino-acid content of individual proteins is very imperfect. Further, it follows from the latest review of Hoffmann and Gortner,[44] that there is no relation between the amount of alkali bound at a given pH and the content of dibasic amino-acid. Consequently it does not seem possible to determine the proportion of terminal groups that are effective in binding acids and alkalis in the individual proteins, and which groups may react with each other by ring formation. Thus it is possible to explain the changes brought about by the action of heat on globulin and seralbumin, by assuming a ring structure formed from the terminal groups, without excluding other changes demonstrable by a different method. Still, this interpretation of the heat-changes does not explain why they are absent in the case of certain proteins. It must remain for further investigations to determine whether the particular chemical constitution of those proteins which are unaffected by heat, may favor the assumption that a ring formation of the terminal groups is entirely absent or occurs upon heating only to a slight extent.

EDITOR'S NOTE.

It is a mistake to think that the behavior of complicated molecular groups must be or can be understood in terms of precise chemical changes. We must squarely face the fact of nature, however unpleasant it may be to some, that as molecular groups increase in size and complexity, their residual fields generally become less and less discrete and invariable. (See P. V. Wells, *J. Wash. Acad. Science,* **9**, 361 [1919].) It appears to the Editor that the apparent anomalies of some of Prof. Spiegel-Adolf's experiments may be resolved by the application of physico-chemical concepts, providing one is untrammeled by the necessity of forcing a definite chemical formula. Here the readjustments of the particulate units involved do not appear to be as precise and definite as occur in what we ordinarily understand to be chemical compounds. See also Chapter I, this volume. *J. A.*

[43] Greenberg, D. M. and Schmidt, C. S A., *Proc. Soc Exp. Biol. Med.,* **21**, 281 (1924).
[44] Hoffmann. W. F. and Gortner, R. A., Colloid Symposium Monograph, Vol. III (1925).

Inorganic Ferments

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THE COLLOIDAL NATURE OF FERMENTS

On approaching the subject of the inorganic ferments (i.e., colloidal enzyme models), we immediately realize that most ferments are either colloids themselves or are associated with colloids. The question of whether or not ferments (enzymes) should be considered colloids can only be answered when they have been successfully purified. Such purification has not yet been accomplished, despite the vast amount of work devoted to this problem, which (thanks to R. Willstätter, H. Euler, Pekelharing and others) has already brought us much nearer the goal. However, as far back as 1899 G. Bredig and R. Müller von Berneck pointed out the colloidal nature of ferments by comparing enzyme action with ordinary inorganic catalytic processes, and even to-day after a twenty-five year advance in the study of ferments, the best students of these phenomena cannot disregard their colloidal nature, or the colloidal nature of their carriers.

E. Duclaux [1] pointed out the colloidal nature of the ferments, and according to Sir Wm. Bayliss [2] "One of the most important physical properties of enzymes is their colloidal nature. . . ." E. Abderhalden [3] says, "A whole series of observations on their behavior can be traced back to the colloidal nature of the enzymes." Even such an investigator as Euler,[4] who has tried his best to disprove the colloidal nature of enzymes, finally yielded, as the following statement indicates: "The conception of an enzyme would surely be incomplete unless the nature of its solution and accompanying characteristics were also understood. The degree of dispersity of the catalyst and the substance to which it adheres, especially protoplasm, seems to play a rôle in many enzymatic processes. Laws which may be established by further study of the kinetics of colloidal particles, will be found in enzyme reactions in some form or other, and many an observation already made stimulates further work in this direction, especially systematic researches on surface reactions.". . . "Furthermore, our present knowledge indicates that it is probable either that many enzymes are of themselves found in colloidal state, or that they are attached to colloids in a most intimate manner. Information as to the behavior of substances with large molecules, that is, of substances of a lesser degree of dispersion, is of the highest significance in investigating and understanding enzymes.". . . S. G. Hedin [5] writes in a similar strain: "From a physical

* Translated by Eleanor G. Alexander, M.A., New York.
[1] Duclaux, E., "Traité de Microbiologie," 2, 96, Paris, 1899.
[2] Bayliss, W. M., "The Nature of Enzyme Action," p. 24, London, 1919; ibid., Naturwissenschaften, 10, 983, Berlin, 1922.
[3] Abderhalden, E., "Lehrbuch der physiolog. Chemie," 4th ed., 2, 284, Berlin, 1921.
[4] Euler, H., Ber., 55, 3590 (1922); ibid., "Chemie der Enzyme" (2nd ed.), 1, 63, München, 1920.
[5] Hedin, S. G., "Grundlagen der physikalischen Chemie in ihrer Beziehung zur Biologie," 2d ed., p. 119, München, 1924.

point of view, enzymes behave like colloids. To speak frankly, we must leave it to the future to decide if this indicates that enzymes are themselves colloids, or that enzymes are always intimately associated with colloidal substances." *

Willstätter [6] also expressed himself as follows: "The colloidal nature of enzymes which governs their physical and chemical properties, is distinctly graded in power and sensitivity from invertin to pancreas-lipase and plant-lipase. The colloidal condition does not determine the activity of invertin, but merely determines whether it remains active. This enzyme when freed from its analytically demonstrated impurities, has the sensitiveness of a colloid weak in electric charges. Loss of colloidal state and disappearance of enzyme activity seem to go hand in hand, and to be mutually dependent. . . . On the other hand the usual reaction of pancreas-lipase is influenced by every change of the colloidal system in which it is embedded. . . . Seed-lipase is either bound by adsorption to an insoluble carrier of the protein group and is influenced in its entire behavior by its incipient hydrolysis (germination or pepsin action); or else the protein substance is the colloidal carrier of the active lipatic group as part of the lipase molecule itself."

R. Willstätter, in his excellent Faraday Lecture (1927), makes the following statement: "We must conceive of an enzyme as consisting of a specifically active group and a colloidal carrier. With the latter, other substances of high molecular weight are connected in variable fashion. The colloidal carrier seems to be somewhat variable, but not unessential for the stability of the active group."

In a recent work on "Ferments" by C. Oppenheimer and R. Kuhn,[7] Willstätter's co-worker, Kuhn, writes (p. 95): "In the majority of ferment reactions . . . ferments are partially in the form of coarse suspension, partially in colloidally dispersed solution. The study of ferments involves systems macro- and micro-heterogeneous in the sense of G. Bredig.[8] Optically clear ferment solutions have not been positively demonstrated. The most highly purified saccharose solutions of R. Willstätter and H. von Euler still show a faint Tyndall effect." In the same book (p. 42) C. Oppenheimer [9] says: "The ferments are substances of high molecular weight and of colloidal structure, electrolytically dissociated as ampholytes, and resemble secondary-valence compounds."

It is true that L. Michaelis and his co-workers have in many cases been able to explain the kinetics of enzyme-reactions with formulæ which were developed from the law of mass action in homogeneous solutions. But it is quite possible that in "microheterogeneous systems" as well, under certain conditions, the suspended particles follow this law. Even Michaelis, after studying adsorption, finally comes to the following conclusion: "It may be that the ferment, even in its seemingly aqueous solution, is not in a state of true solution, but can be considered as a microheterogeneous phase in the sense of Bredig."

* If enzymes contain specific active surface groups of molecules, which cannot function in the absence of a protective, kinetically active, and activating colloid carrier, then *only the combination* may be justly regarded as an enzyme. Neither an arrow head, a feather nor an arrow shaft is an arrow, though the three, when properly joined, make an arrow and function as an arrow. *J. A.*
  [6] Willstätter, R., *Ber.*, **55**, 3609 (1922).
  [7] Oppenheimer, C. and Kuhn, R., "Die Fermente und ihre Wirkungen," 5th ed., p. 95, Leipzig, **1924.** *Naturwissenschaften*, **15**, 585 (1927); **14**, 937 (1926); *Z. physiol. Chem.*, **151**, 1 (1926).
  [8] Bredig, G., *Biochem. Z.*, **6**, 283 (1907).
  [9] See Oppenheimer, C. and Stern, E., "Handbuch der Biochemie," 2nd ed., Jena, **1923.**

FERMENTS AS CATALYZERS

The conception of the ferments as catalyzers goes back to J. Berzelius.[10] To Wilhelm Ostwald (1894)[11] especially, we owe the clear understanding that catalysis is a change in the speed of reaction through the presence of a substance known as a catalyzer. It took a long time for these ideas on enzyme action to be recognized and taken up by the scientific world, although Ch. F. Schönbein[12] had described the decomposition of hydrogen peroxide through the catalytic action of platinum as "the prototype of all fermentation." The composition and specific nature of the ferments were long held doubtful.[13] Many investigators were skeptical of catalysis, especially in ferment chemistry, up to comparatively recent times. A change in this opposition came but slowly, after G. Bredig (1899)[14] began to build up models of ferment action in which he studied catalyzers of known chemical composition and in the colloidal state. He pointed out that in these systems we find many of the phenomena observed in ferments. These "model systems" first investigated by him and his co-workers R. Müller von Berneck, K. Ikeda and W. Reinders, were the colloidal metal sols which he called "inorganic ferments."

PRODUCING METAL SOLS ELECTRICALLY

If the catalytic action of metal sols is to be observed without interference, the sols must be free from impurities. On this account all chemical methods were excluded. Bredig produced metal sols by the electrical dispersion method invented by him,[15] which consists in electrical cathode-dispersion of the metal under water.

According to the simplest form of this method, an ammeter A (Fig. 1), a rheostat W, and 2 electrodes consisting of wire about 1 mm. thick of the metal to be dispersed, are successively put into circuit with the poles, K, of a source of direct current of 110 volts. One electrode is covered by a narrow glass tube to insulate it so that it can be handled. The rheostat is so set that by short circuiting and carefully separating the electrodes under water, there is produced a small arc about 1 mm. long, which has about the desired strength (cf. below). The experiment can be carried out using a dish, S, cooled outside with ice, of about 50-100 ccm. capacity, filled with very pure distilled water free from carbonic acid (specific electrical conductivity $2-3.10^{6} \frac{cm.}{ohm}$

The wire electrodes are placed in the position indicated in Figure 1, showed 1-2 cm. under the level of the water, short circuited, and then are separated slowly about 1-2 mm. apart, which establishes a small arc. As long as this arc burns quietly, the colloidal metal can be seen issuing forth from the cathode in dark clouds and spreading out in the surrounding fluid, partly as a sol, and partly as coarser particles. The arc is very easily broken, in which case, the short circuit is made again to recommence the action; continuous stirring

[10] Berzelius, J., *Jahresbericht Chem.*, 15, 237 (1836).

[11] Ostwald, Wilh., "Ueber Katalyse," Ostwald's Klassiker d. exakt. Wissensch., Nr. 200, p. 15 et seq., Leipzig, 1923.

[12] Schönbein, Ch. F., *J. prakt. Chem.* (1), 89, 335 (1863).

[13] For accompanying literature and criticism, see Oppenheimer, C. and Kuhn, R., *loc. cit.*, pp. 13 and 14.

[14] Bredig, G., and Müller von Berneck, R., *Z. physik. Chem.*, 31, 258 (1899). Bredig, G. and Ikeda, K., *ibid.*, 37, 1 (1901). Bredig, G. and Reinders, W., *ibid.*, 37, 323 (1901); Galecki, A., *Polnische Akadem. d. Wissensch. A.*, p. 93, 1925.

[15] Bredig, G., *Z. angew. Chem.*, 11, 951 (1898); *ibid.*, "Anorganische Fermente," Habilitationsschrift, p. 22, Leipzig, 1901.

is kept up until the water in the dish has become deeply colored. In place of the hands, there is also a very convenient apparatus [16] for handling and moving the electrodes in the manner described, in which the electrodes are fastened and moved more accurately by screws. The strengths of current to be used are: platinum and silver 4-12 amp., gold and palladium 8-10 amp., iridium 10-30 amp., with gold, palladium, and iridium stable sols are formed if a trace of alkali (about $M/1000$ NaOH per liter) is added to the water beforehand. As to the stabilizing rôle of the alkali, see for example H. T. Beans and E. H. Eastlack.[17] * In general, sols produced with weaker current
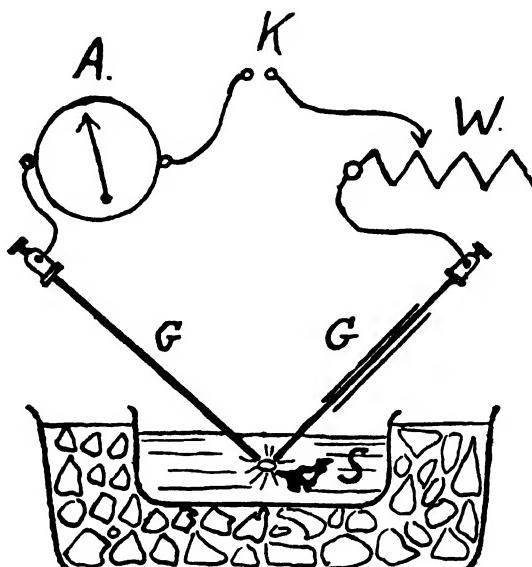
Fig. 1.—Electric Dispersion Apparatus.

are more stable than those produced with stronger current. The electrically dispersed deeply colored fluids contain besides the ultramicroscopic particles, coarser ones as well, which can be separated from the sol by filter paper. Such filter paper can not be used too long, and must be frequently renewed because the pores become clogged by adsorption, and the sol no longer passes through unchanged. Metal sols made in this way may also contain some oxide. The Svedberg [18] used for producing metal sols the oscillatory discharge of an inductor system containing Leyden jars and induction coils, which are of advantage in organic dispersing media, as the separation of carbon is thus avoided.

[16] Koehler, Fritz., Univ. Mechaniker Leipzig Windscheidstr. 33 Katalog uber "Kolloid-Chemie-Apparate."
[17] Beans, H. T. and Eastlack, H. E., *J. Am. Chem. Soc.*, **37**, 2667 (1915).
* While there is no doubt as to the *stabilizing* action of some electrolytes, the view that they are *essential* to colloid *formation* seems erroneous. Thus C. Harvey Sorum of the University of Wisconsin (*J. Am. Chem. Soc.*, 1928) prepared ferric hydroxide sols showing no trace of Cl. *J. A.*
[18] The Svedberg, "Methoden zur Herstellung kolloider Lösungen," Steinkopff, Dresden.

The Inorganic Sols as Catalyst Models

It has been known for a long time (Thénard, 1818), that many metals, especially the precious metals, reduce hydrogen-peroxide according to the general equation: $2H_2O_2 = 2H_2O + O_2$. Berzelius and Schönbein had, as before mentioned, pointed out the analogy between these catalyzers and ferment action. In order to study inorganic sols as "ferment models," G. Bredig brought the catalyzing metals into the state of colloidal sols, in which condition many ferments are found, especially the so-called "catalases"—i.e., those ferments which decompose hydrogen peroxide into water and oxygen. He and his co-workers in the study of the catalytic action of metal sols on hydrogen peroxide, noticed in many cases a strong resemblance between these and the catalases of organisms. Bredig therefore named these sols *"inorganic ferments."* However, he emphasized the fact that this does not mean an identity with the ferments, but merely a model-like analogy which at any rate goes surprisingly far, and both series of phenomena must have a common basis.

Most striking is the extraordinarily minute quantity of the colloidal metal which can still be detected by its catalytic action on H_2O_2. Colloidal platinum in a dilution of 1 gram atom in 70.10^6 liters of water, gold 1 gram atom in 10^6 liters, and palladium 1 gram atom in 26.10^6 liters of water still gave a noticeable catalytic reaction on an incomparably greater quantity of H_2O_2. In this respect these sols bring to mind enzyme solutions [19] which exhibit a similar quantitative relationship between catalyser, substrate, and solution.

As is known from the work of Thénard and Schönbein, minute traces of other substances also have a catalytic effect on hydrogen peroxide. According to Bredig and Müller von Berneck [14] (*loc. cit.* p. 278, 292) MnO_2 in alkaline fluid reacts in a dilution of 10.10^6 liters, Co_2O_3 in 2.10^6 liters, CuO_2 in 10^6 liters, and PbO_2 in 10^5 liters per mol. Iron compounds react similarly even when more highly diluted. Here, also, we are dealing with a colloidal suspension, even though the actual reaction-mechanism can, perhaps, be referred back to the intermediary formation of soluble higher oxidation products of the metals. For outstanding studies on these subjects we are indebted to G. v. Elissafoff, E. Spitalsky, L. van Bohnson, A. C. Robertson, V. Zotier, F. Mayer, W. H. Schramm, F. G. Tryhorn, and G. Jessop, and others.[20] Even so, the relationships between these substances is very obscure. G. Bredig and Müller von Berneck have tried to explain them by the formation and decomposition of higher degrees of oxidation of the metals concerned.

We know that the enzyme catalase is destroyed by too high a concentration of hydrogen peroxide. Similarly colloidal silver, which exerts a strong catalytic action on H_2O_2, is thrown out of solution by this substance and rendered inactive, according to the observations of D. McIntosh, if the liquid is not sufficiently alkaline. Similar observations were made by G. Bredig and A. Marck on colloidal manganese dioxide.

The kinetic time law governing the decomposition of hydrogen peroxide

[19] See Oppenheimer, C., and Kuhn, R., *loc. cit.*, p 355.
[20] Elissafoff, G. v., *Z. Elektrochem.*, 21, 352 (1915). See also Freundlich, H., "Kapillarchem., 3rd ed., p. 306. Spitalsky, E. and Petin, N., *Z. physikal. Chem*, 113, 161 (1924), Cf. also the older literature of J. von Bertalan, J. Duclaux and others on H_2O_2 catalysis by iron compounds, van L. Bohnson and A. C. Robertson, *J. phys. Chem*, 25, 19 (1923); *J. Am. Chem. Soc.*, 45, 2493, 2512 (1923); 47, 1299; see also A. K. Goard and E. K. Rideal, *Proc Roy. Soc. (London) A*, 105, 148; Kimura, Taizo, *J. Biochem.*, 3, 211. Zotier, V., *Bull. Soc. Chim. France* (4), 13, 61 (1913); 15, 402 (194); Bellucci, L. and Paravano, N., *Chem. Centralbl.* 1, 222, 528 (1907); Tryhorn, F. G. and Jessop, G., *J. Chem. Soc. (London)*, 127, 1320 (1925). Mayer, F. and Schramm, H. W., *Z. analyt. Chem.*, 56, 129 (1917); Quartaroli, A., *Gazz. chim.*, 54, 713 (1924); 55, 252, 264, 619 (1925). Clarens, J., *Bull. Soc. Chim. France* (4), 33, 280 (1923).

by platinum as catalyst, approximates a reaction of the first order—i.e., the velocity of decomposition of H_2O_2 is proportional to its concentration. G. Senter [23] found the same thing for blood catalase, as is shown in the following table:

Blood Catalase (Senter).			Colloidal Platinum (Bredig and Müller von Berneck).		
Time	$C_{H_2O_2}$	0.4343 k	Time	$C_{H_2O_2}$	0.4343 k
0	39.7	.....	0	47.4	.....
5	32.2	0.0175	10	37.9	0.0097
10	26.7	0.0163	20	30.0	0.0099
20	17.8	0.0176	30	23.6	0.0101
30	11.6	0.0185	40	18.2	0.0104
50	4.8	0.0191	60	11.0	0.0106

Here the time is given in minutes and the concentration $C_{H_2O_2}$ in ccm. of permanganate, and k stands for the velocity constant of the first order. A similar time law approximating the first order is also shown by the catalytic decomposition of H_2O_2 by electrically made solutions of palladium according to Fortner,[24] and iridium according to Brossa.[25] In very many instances k shows a true progression—in the sense that k increases as the reaction progresses and the concentration of H_2O_2 decreases. It is held by most investigators (D. A. MacInnes, H. G. Denham, H. Freundlich [26]) that the reaction speed is proportional to the amount of H_2O_2 adsorbed at the surface of the colloidal particles, whereby this increase of the constant of the first order is explained. In the case of silver (McIntosh [21]) and manganese dioxide (Marck [22]) there is, upon continuing the reaction, a decrease in constants of the first order if the fluid is not sufficiently alkaline. This may be explained by chemical solution and destruction of the catalyzer by H_2O_2, which in these cases was clearly demonstrated. Very similar deviations from the scheme of a first order reaction have been found in the ferment chemistry of catalase,[27] in the sense that these constants increase with continued reaction and decreasing H_2O_2 concentration, as also in many cases in the reverse sense that too high an H_2O_2 concentration destroys the catalase. P. Waentig and O. Steche [28] consider the deviations in the time action of catalase from the velocity of a reaction of the first order, to be consequent on adsorption processes which they believe affect the reacting substance, H_2O_2, as well as the molecular oxygen which appears as a reaction product.

The rate of catalytic action in "microheterogeneous" systems (colloidal platinum) was compared with that in "macroheterogeneous" systems (platinum

[21] McIntosh, D., *J. Phys. Chem.*, 6, 15 (1902); Santesson, C. G. G., *Skandin. Archiv. Physiol.*, 44, 262 (1923).
[22] Bredig, G., and Marck, A., "Gedenkboek van Bemmelen," p. 342. Te Helder, Niederlande, 1910.
[23] Senter, G., *Z. physik. Chem.*, 44, 257 (1903); 51, 673 (1905). Senter, G., *Proc. Roy. Soc.*, 74, 201, 516 (1904-5). Rona, P., Feigel and Nakahara, *Biochem. Z.*, 160, 272 (1925). Warburg, O., *Naturwissenschaften*, 14, 759 (1926). Blaschko, H., *Biochem. Z.*, 175, 68 (1926). v. Euler, H. and Josephson, *Liebig's Ann.*, 455, 1 (1927).
[24] Bredig, G., and Fortner, M., *Ber.*, 37, 798 (1904).
[25] Brossa, G. A., *Z. physik. Chem.*, 66, 162 (1909).
[26] Denham, H. G., *Z. physik. Chem.*, 72, 686 (1910). Freundlich, H, "Kapillarchemie," 3rd ed., p. 673. MacInnes, D. A., *J. Am. Chem. Soc.*, 36, 878 (1914).
[27] Vergl. Senter, *loc. cit.*, and further literature by C. Oppenheimer and R Kuhn, *loc. cit.*, p. 355 et seq.
[28] Waentig, P. and Steche, O., *Z. physiol. Chem.*, 72, 226 (1911); 79, 446 (1912); *Biochem. Z.*, 60, 463 (1914).

foil) especially by Bredig and Teletow [29] with respect to the formation of oxygen from aqueous hydrogen peroxide solution, and by J. W. McBain and K. Jablezynski [30] on the formation of oxygen from aqueous chromous chloride. In both cases it is evident that in macroheterogeneous systems corresponding to the Nernst Theory, diffusion phenomena are determining factors for the rate of reaction. Stirring has an influence here, but not in a microheterogeneous system. The temperature of the microheterogeneous system is considerably larger than that of the macroheterogeneous system which fact accords with the Brownian movement of colloidal particles. St. Ruszyniak [31] discovered the surprising fact that gold sols of a smaller degree of dispersion exert less catalytic action than those of higher degrees of dispersion. Yet it is questionable whether his blue and red sols can be compared directly with one another, since they were not prepared electrically, but chemically, and due to their method of preparation, contained impurities. Observations of Bredig and Müller von Berneck (*loc. cit.*, p. 306),[14] J. Eggert and Erich Müller and others, show further that catalytic action is dependent on the degree of dispersion of the catalyst. There are, however, other influences than those of dispersion (Galecki),[14] such as adsorption, oxidation, etc.

Bredig [15] (*loc. cit.*, pp. 93, 94) held the view that the oxygen given off in the metallic catalysis of H_2O_2 is effective either in free molecular form dissolved in the metal, or else is chemically bound in a metal oxide of the formula M_xO_y. The question, however, is answered by experiments of E. B. Spear,[32] which show that increase in oxygen pressure in the proportion of $1:200$ atmospheres does not noticeably increase the catalytic action of colloidal platinum, palladium, iridium, gold, and silver solution in the decomposition of H_2O_2. Thus the *free* oxygen is not of fundamental importance in the process. Therefore only chemically bound oxygen is active here. Also supporting this oxide theory are observations by J. Weinmayer, E. Wilke, and A. v. Antropoff [33] on the intermediate, often pulsating structure of a solid skin in the catalytic action of mercury on the decomposition of H_2O_2. Furthermoie, A. de Gregorio Rocasolano [34] says that the action of the platinum sols as catalysts is stronger the higher their "active" oxygen content, that is, oxygen which liberates its equivalent of iodine from acid potassium iodide solution, and which is not free, but chemically combined as a platinum oxide.* H. Wieland,[35] on the other hand, regards the catalytic action of metals and catalases as a transfer of hydrogen from one molecule of H_2O_2 to another. Bredig and Müller von Berneck (*loc. cit.*, p. 293) [14] had already pointed out the fact that the development of O_2 from H_2O_2 through reduction of a higher oxidation product of the catalyst, formed by reduction of the first molecule of H_2O_2, can be effected by a second H_2O_2 molecule. They had also partially explained the extraordinary dependence of these reactions on the concentration of the hydroxyl-hydrogen ions (pH of Sörensen and Michaelis).

[29] Bredig, G and Teletow, J., *Z. Elektrochem.*, **12**, 581 (1906). See also Senter, G., *J. physik. Chem.*, **9**, 311 (1905). Sand, H. J. S., *Proc. Roy. Soc. (London)*, **74**, 356 (1905), *Z. physik. Chem.*, **51**, 64 (1905).
[30] McBain, J. W, Dissertation, Heidelberg, 1909; Jablezynski, K., *Z. physik. Chem.*, **64**, 748 (1908).
[31] Ruszniak, St, *Z. physik. Chem.*, **85**, 681 (1913).
[32] Spear, E. B., *J. Am. Chem. Soc.*, **30**, 195 (1908).
[33] Bredig, G. and Weinmayer, I., *Z physik. Chem.*, **42**, 601 (1903); Bredig, G. and Wilke, E., *Verh. Naturhist. medic. Vereins Heidelberg N.F.*, **8**, 165 (1905). v. Antropoff, A., *Z. physik. Chem.*, **62**, 52, 79 (1908); *J. prakt. Chem.*, **77**, 273 (1908).
[34] Rocasolano, A. de Gregorio, *Nachricht. Gesellsch. Wissensch. Gottingen. Math. phys. Kl.*, 177 (1924). See also Adams, Royer and Shriner, R. L., *J. Am. Chem. Soc.* **45**, 2171 (1923).
* See paper by A. de G. Rocasolano in Vol I of this series. *J. A.*
[35] Wieland, H., *Ber.*, **54**, 2353 (1921); **55**, 3647 (1922).

The catalytic action of metal sols as well as that of natural catalase enzyme is markedly affected by *the concentration of hydroxyl ions,* i.e., by what is now known as pH. Colloidal gold, palladium, silver, and mercury give only very slight action in acid or neutral medium in the decomposition of H_2O_2 into water and oxygen; but on the other hand, are very active in alkaline solution. The same holds true for colloidal oxides of manganese, lead, copper, and cobalt, etc. Here the particular fact emerged that adding alkali to platinum,[14] gold,[14] palladium,[24] and manganese[22] dioxide sols, the *time law of a first order* reaction for decomposition of H_2O_2 completely *disappeared,* and often, as the reaction proceeded, a marked increase in the constant of the first order appeared. This change in the degree of reaction is, according to Calvert, partly caused by the fact that the hydrogen peroxide forms salts with alkalis in aqueous solution.[36] Common to all the above-mentioned sols is the peculiar characteristic that increase in the amount of alkali (i.e., increase in pH) enormously increases the action in the decomposition of H_2O_2. Thus
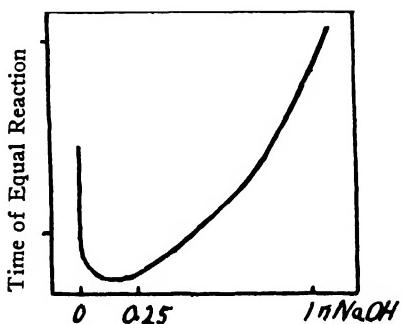
Fic. 2.—Platinum Colloid.   Fig. 3.—Mandelic Catalase.
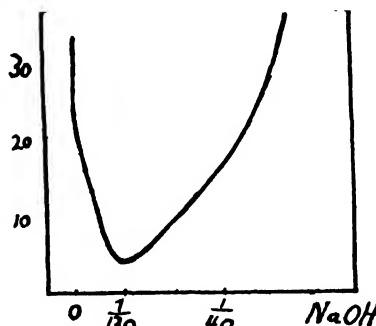
the hydroxyl ions play the same rôle here as the "activators" (or "promotors") in enzyme chemistry. However, with still further addition of alkali, the catalytic action finally reaches a maximum. It is a striking fact that a similar influence of increased alkali (i.e., of pH) with a maximum, is also found in the natural catalase enzymes[37] as is shown in Figures 2 and 3.

Strange to say, this marked influence of alkalis with its maximum, does not exist in the catalytic decomposition of H_2O_2 by iridium sol, according to investigations of G. A. Brossa.[25] Even in basic solution, the time law of iridium sol remains approximately that of a first order reaction. On the other hand, most acids, such as sulfuric and nitric, cause a marked increase in catalytic action of the iridium sol, according to Brossa. And strangely enough, these acid reactions seem also to go through a maximum.

The influence of the amount of the catalyst in these sols on the decomposition of H_2O_2 has also been investigated. Whereas with iridium (Brossa[25]) the catalytic action is nearly proportional to the concentration of the catalyst, with platinum (Berneck, Ikeda[14]) and gold (Reinders[14]) the velocity increases much more rapidly than the concentration. Data on natural catalases vary. According to G. Senter,[23] the action of blood catalase in very dilute

[36] Calvert, H. Th., *Z. physikal. Chem.,* **38,** 513 (1901).
[37] Oppenheimer, C. and Kuhn, R., *loc. cit.,* p. 359.

H_2O_2 solutions is proportional to the enzyme concentration. Other authors assert that the catalytic action increases more quickly than the enzyme concentration. In concentrated H_2O_2 solutions with a relatively small amount of enzyme, there is a noticeable reversal of the action, for the enzyme is destroyed during the reaction so that the kinetics depend on the proportion between the amounts of enzyme and H_2O_2.[38] This was also the case with labile inorganic catalysts such as silver. With silver sol (McIntosh[21]) as in the literature on catalase, the statement is found that under certain conditions, a definite amount of catalyst decomposes a definite amount of H_2O_2, which is comprehensible by virtue of the great liability of very dilute neutral silver sol and catalase, when acting on H_2O_2.[39, 40]

With the natural enzymes, the influence of an increase in temperature is a complication. For on the one hand the catalytic action of the enzyme increases with rising temperature; on the other hand there appears a spontaneous inactivation of the enzyme with rising temperature, and in fact, the higher the temperature, the more quickly this appears. Both results of the rise in temperature overlap one another, and thus with increase in temperature the enzyme action goes through a maximum. Similarly the activity of enzyme solutions are often dependent upon temperature and duration of the pre-heating.

C. von Ernst[41] found such a maximum for the catalytic action of platinum sol on oxy-hydrogen gas. In the action of platinum sols on H_2O_2, G. Bredig and Müller von Berneck[14] did not find any maximum; however they did find a result of longer pre-heating, especially in the presence of minute amounts of electrolytes.

Since the excellent early work of Schönbein,[42] it has been known that the catalytic properties of the natural catalase enzymes are inhibited by the minutest traces of certain substances such as H_2S or HCN. More recent literature[43] contains many examples of such inhibition or "poisoning" of the enzyme reaction by traces of certain substances. G. Senter[28] and Hata[44] in particular have thoroughly studied the inhibitive action of such "poisons" on the activity of catalase enzymes.

In a strikingly similar way, strongly inhibitive "poisoning" actions also appear upon adding traces of certain substances to inorganic catalyzers. M. Faraday[45] had already described the fact that oxy-hydrogen gas containing H_2S or CS_2 as impurities, is no longer catalyzed by platinum foil. G. Bredig and his co-workers have now shown that the metal sols are checked in their catalytic action on H_2O_2 by minute traces of certain substances, in a similar manner to catalase enzymes. The following table of G. Senter shows the striking similarities of these so-called "poisonings" of natural catalase and metal sols. The concentrations are given in mols per liter of the "poisons" which lowered the catalytic action of blood catalase and platinum sol to one half their original activity. The studies on the poisoning of platinum sol owe their origin mainly to a thorough piece of work done by G. Bredig and K. Ikeda.[14] Similar "poisonings" were investigated in gold sols by W. Rein-

[38] See Oppenheimer, C. and Kuhn, R., *loc. cit.*, p. 355 *et seq.*
[39] Bredig, G., "Katalyse," in Ullmann's "Encyclopaedie der Techn. Chemie," 5, 670 (1919).
[40] See Oppenheimer and Kuhn, *loc. cit.*, p. 359
[41] Ernst, C., *Z. physikal. Chem.*, 37, 477 (1901).
[42] Schonbein, Ch. F., *J. prakt. Chem.* (1), 105, 202 (1868).
[43] See Oppenheimer and Kuhn, *loc. cit.*, 63-77. Euler, H., "Chemie d. Enzyme," 2nd ed., 1, 134.
[44] Hata, S., *Biochem. Z.*, 17, 156 (1909).
[45] Faraday, M., *Ostwald's Klassiker*, 87, 30.

ders,[14] in palladium sols by M. Forstner,[24] in iridium sols by G. A. Brossa,[25] in silver sols by D. McIntosh [21] and C. G. Santesson.[21]

Poison	Platinum Sol (Bredig and Ikeda)	Hemase (Senter)
H_2S	1/350,000 molar	1/1,000,000 molar
HCN	1/20,000,000 "	1/1,000,000 "
$HgCl_2$	1/2,500,000 "	1/2,000,000 "
$HgBr_2$	....	1/300,000 "
$Hg(CN)_2$	1/200,000 "	1/300 "
I (in KI)........................	1/7.000,000 "	1/50,000 "
$NH_2OH.HCl$	1/25,000 "	1/80,000 "
Phenylhydrazine	....	1/20,000 "
Aniline	1/5,000 "	1/400 "
Arsenic acid	1/50 "	No inhibition at 1/2,000
CO	Very poisonous	No inhibition
HCl	1/3,000 molar	1/100,000 molar
NH_4Cl	1/200 "	1/1.000 "
HNO_3	No inhibition	1/250,000 "
H_2SO_4	" "	1/50,000 "
KNO_3	" "	1/40,000 "
$KClO_3$	Almost no inhibition	1/40,000 "

The table shows that whereas some substances affect blood catalase (hemase) in the same way as they do platinum sol, in other cases, especially as far as concerns acids, salts with oxidizing properties, and carbon monoxide, the behavior of the two kinds of catalysts may be very different.

As for other metal sols as well, "poisons" are not always the same. Iodine, very inhibitive for platinum, scarcely affects iridium in higher dilutions. In drawing an analogy between the action of catalase enzyme and metal sols on H_2O_2, all substances which are "poisons" for the enzyme do not necessarily behave similarly toward metal sols and *vice versa*. The same substances do not always behave as poisons toward both inorganic catalysts and enzymes. For example, CO in most cases acts as a powerful poison on platinum catalysis (with oxy-hydrogen gas, H_2O_2). According to Warburg [23] CO markedly inhibits the respiration of yeast, but it does not poison H_2O_2 catalyzed by MnO_2. Prussic acid is "poisonous" to catalase, but activates the enzyme papain. Poisoning depends throughout on the nature of the catalysts and the substrates. Nevertheless, the entire behavior of enzymes and metal sols with regard to inhibiting substances is often analogous down to the smallest details. The objections raised by J. H. Kastle and A. Loevenhart,[46] and by Th. Bokorny against this conception, were answered by Bredig [47] and Senter.[23] The analogy is clearly seen in the figures given which demonstrate the rate of H_2O_2 decomposition with natural pancrease catalase (Fig. 4) and with colloidal platinum sol (Fig. 5) with and without (1/∞) the addition of prussic acid. This markedly lowers the catalytic action of blood catalase [23] and platinum sol [14] in molar dilutions of several million liters. The inhibiting action of H_2S, $HgCl_2$, iodine, and other substances upon catalase is noticeable in similar, often astonishingly minute concentrations.

Both enzymes and "inorganic ferments" (metal sols) have the capacity of "recovery" from specific inhibitions, i.e., regenerating either of their own

[46] Kastle, J. H. and Loevenhart, A. S., *Am Chem. J.*, **29**, 563 (1903). Bokorny, Th., *Centralbl. Bakt.* Abt. II, **18**, 737 (1907).
[47] Bredig, G., *Centralbl. Bakt.* Abt. II, **19**, 485 (1907).

accord or with the aid of specific "counter poisons." Catalase (Senter [23]) and platinum sol (Bredig and Müller von Berneck, and K. Ikeda [14]) which have been poisoned by prussic acid, can be reactivated for H_2O_2 by blowing in air. Reactivation of metal sols poisoned with HCN can also be attained on standing with H_2O_2 (cf. curves of "recovery" in Figures). According
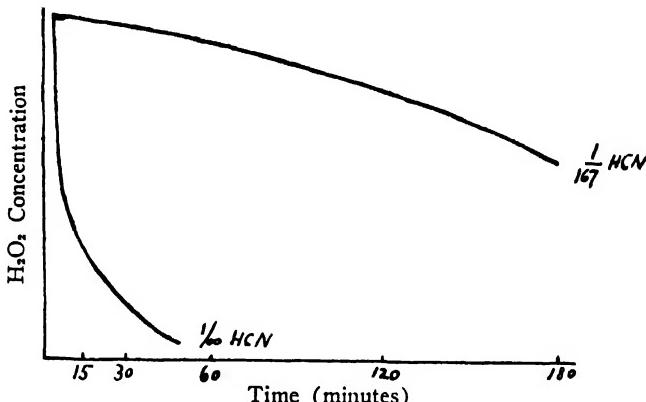
FIG. 4.—Catalase of Pancreas, Poisoned by HCN.

to E. Buchner,[48] fermentation of sugar with zymase is checked by prussic acid and restored again by blowing in air. Platinum sol or hemase preparation poisoned with mercuric chloride, will not become active again of its own accord. There are reversible and irreversible poison reactions [49] with inorganic catalysts as well as with enzymes. The catalytic action of platinum or palladium sol on H_2O_2 can be poisoned by a very minute amount of carbon monox-
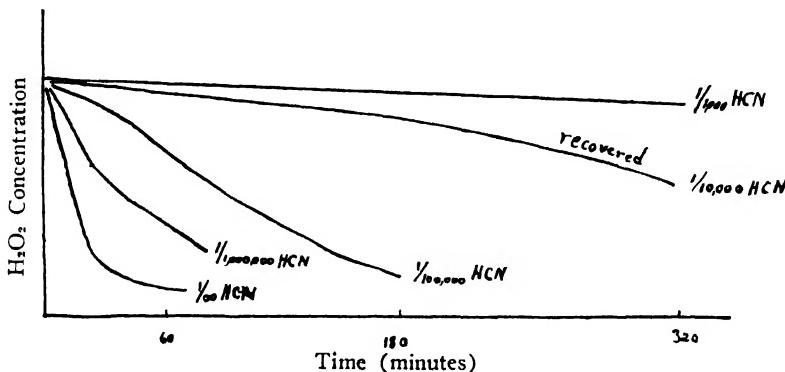
FIG. 5.—Colloid of Platinum, Poisoned by HCN.

ide, provided that the CO is put in contact with the metal sol before the addition of H_2O_2. Even so, after a time the catalyst "recovers" itself, while the carbon monoxide becomes oxidized by the H_2O_2 added. In the end, the metal sol becomes more active than it was before having been "poisoned" (Bredig and K. Ikeda [14]) (M. Fortner [24]) (cf. Fig. 6).

The order in which the catalysts (or enzymes), substrate, and poison are

[48] Buchner, E., *Ber.*, **30**, 2672 (1897). Warburg, O., *Biochem. Z.*, **165**, 196 (1925).
[49] See also Euler, H., "Chemie d. Enzyme," 2nd ed., 134-162, 1920.

mixed is often of the greatest importance for the action of inhibiting or activating agents. The "poisoning" action of prussic acid on blood catalase is greater when the "poison" is added before the H_2O_2 solution, than when the acid is added last to a mixture of enzyme and H_2O_2. The same is true of platinum sol (Berneck, Ikeda [14]).

There are a number of possible explanations for these inhibiting reactions. The reagents might bind the catalyst in a purely chemical way and thus change it into an inactive form. This explanation is very probable for the action of prussic acid on metal sols and on catalase enzyme. It is also possible that the surface of the "microheterogeneous" catalyst particle is covered
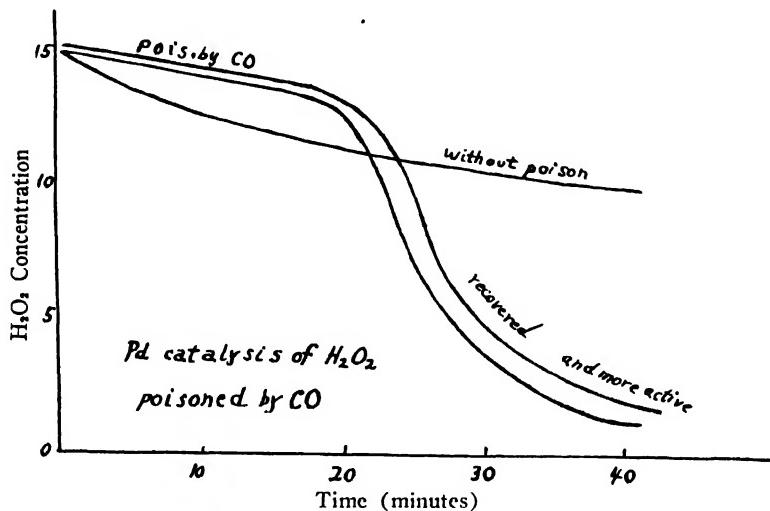
Fig. 6.—Palladium Catalysis of H_2O_2 Poisoned by CO.

over as a result of chemical reaction, by a catalytically inactive layer of foreign substance. Perhaps $HgCl_2$ or H_2S acts this way with platinum particles, whose surface becomes covered with Hg_2Cl_2 or sulfur. A third way in which substances "poisonous" to catalysts might act is that they may become bound to the surface of the catalysts by adsorption, and thus crowd out the substrates (cf. Bredig and Ikeda [14]). Bredig and Ikeda also showed that the poisoning action of many substances on the platinum catalysis of hydrogen peroxide, depends on the concentration of the poison according to an equation which H. Freundlich was actually able to deduce from his well known adsorption isotherm.[50] The poisoning of platinum catalysis of H_2O_2 by iodine took place at $25°$ according to Bredig and Ikeda,[14] as shown in the following tables (concentration of platinum 2 mg. per liter):

Concentration of iodine in micromols	0.0	0.1	0 2	0.4	1.0	2.0	4.0
Time to reduce activity one half							
Observed	7.7	13.9	19.8	21.6	33.8	48.4	65.7 minutes
Calculated	...	13.9	17.2	22.2	33.0	46.4	66.7 "

[50] Freundlich, H., "Kapillarchemie," 3rd ed., 682, 1922.

The researches of O. Meyerhof also support adsorption as an explanation of poisonings.[51] According to him, the catalytic action of platinum sol on H_2O_2 is considerably delayed by capillary-active substances such as alcohol, ketone, and urethane. This "poisoning" is reversible, and Traube's rule holds good, according to which the action of capillary-active substances increases in a homologous series so that, for example, a lesser concentration of amyl alcohol is just as "poisonous" as ethyl alcohol. It is not impossible that in the investigations of C. H. Neilson and O. P. Terry [52] with catalase-containing kidney extracts, the inhibiting action of hypnotics such as urethane, chloral hydrate, etc., can be similarly explained. Also in a like manner so-called protective colloids (J. Groh [53]) diminish the catalytic action of platinum sol, and indeed, the more colloid present, the more coagulation is inhibited.

Dispersed Metals as Dehydrogenators [54]

It has long been known that hydrogen is given off in the presence of microorganisms, especially in fermentation or decay of organic substances. For example L. Popoff and F. Hoppe-Seyler [55] showed that in certain fermentations formic acid decomposes into hydrogen and carbonic acid. In 1847 Deville and Debray observed that the same reaction took place under the influence of rhodium sponge:

$$H.COOH = H_2 + CO_2$$
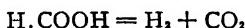
This catalytic evolution of hydrogen from formic acid with rhodium was more intensively studied in Bredig's laboratory by Th. Blackadder.[56] The metal is not active under all conditions, but only when very finely subdivided as rhodium sponge. Even in this state it is often inactive. It can, however, be activated in most cases by oxygen, hydrogen peroxide or sulfur. After activation with sulfur, the catalytic decomposition of formic acid produces a mixture of hydrogen and carbon dioxide which contains traces of hydrogen sulfide. The time law of this decomposition of formic acid is, according to Blackadder, such that the reaction speed is proportional to the formic acid adsorbed on rhodium sponge (according to the Freundlich adsorption-isotherm). Thus the logarithm of the reaction speed is proportional to the concentration of the formic acid in solution. The reaction is considerably hastened by the addition of sodium formate, but comes to a stop in alkaline solution. Here, too, we have a clear case of the influence of H-ion concentration, with a maximum. The effect of stirring is very slight; the reaction speed doubles for each degree rise in temperature. This shows that the speed of this reaction (to distinguish it from the H_2O_2 decomposition referred to above) is determined by the chemical action on the surface, and only slightly by the much quicker diffusion. Similar facts were found by A. Sieverts and E. Peters [57] in the evolution of hydrogen from aqueous sodium hypophosphite

[51] Meyerhof, O., *Pflügers Arch. Physiol.*, 157, 307 (1914). Freundlich, *loc. cit.*, p. 681.
[52] Neilson, C. H. and Terry, O. P., *Am. J. Physiol.*, 13, 427; 14, 248 (1905).
[53] Groh, J., *Z. physik. Chem.*, 88, 414 (1914). McBain, Dissert., Heidelberg, p. 57, 1909. Rideal, E. K., *J. Am. Chem. Soc.*, 42, 749 (1920).
[54] Regarding dehydrogenation by catalysis see Sabatier, P., "La Catalyse en chimie organique," Paris, 1913; Rideal, E. K. and Taylor, H. S., "Catalysis in Theory and Practice," London, 1919; also the papers of Berthelot, M., Ipatiew, *Ber.*, 58, 4 (1925); Knoevenagel, F., *Ibid.*, 36, p. 2837 (1903); Zelinsky, N. D., *ibid.*, 58, 1298 (1925); Palmer, W. G. and Constable, F. H., *Proc. Roy. Soc. (London)*, A, 107, 255, 270 (1925). Adkins, H., and Perkins, P. P., *J. Am. Chem. Soc.*, 47, 1163 (1925), etc.
[55] Popoff, L., Archiv. Physiol., 10, 113 (1875); Hoppe-Seyler, F., *ibid.*, 12, 11 (1876).
[56] Blackadder, Th., *Z. physik. Chem.*, 81, 385 (1912).
[57] Sieverts, A. and Peters, E., *Z. physik. Chem.*, 91, 199 (1916).

solution on catalysis by platinum. Colloidal platinum reacted much more strongly than an equal weight of palladium sponge. Here, also, the catalysis was poisoned by potassium cyanide. J. W. McBain [30] found more complicated relations in the catalytic evolution of hydrogen from acid aqueous chromous chloride solution with platinum sol.

Later researches of E. Müller [58] showed that other platinum metals, especially osmium, also decomposed formic acid catalytically. As the behavior of the "inorganic ferments" indicates, metals exercise on formic acid a catalytic strength proportional to the degree of dispersion. Thus all conditions favoring the colloidality of osmium increase its activity. Metals are most highly dispersed and consequently most active as catalysts, according to Müller, when they are freshly freed from a chemical compound directly in the formic acid solution. This also explains why rhodium was most active, according to Blackadder, when it initially contained its oxide or sulfide. E. Müller could keep the osmium dispersed for a longer time by adding a protective colloid such as gelatin, and thus preventing coagulation and inhibition of the catalysis. Palladium, platinum, ruthenium and iridium as metal sponges also decompose formic acid catalytically, and indeed, the finer their degree of dispersion the better they work. When not sufficiently dispersed, platinum, iridium, and ruthenium, according to E. Müller, show practically no catalytic activity at all.

In a later paper E. Müller and F. Müller [59] show that platinum metal in sufficient dispersity can also decompose formaldehyde catalytically. Osmium works best, evolving CO_2 and H_2 and some CO; with rhodium, ruthenium, and palladium a rather large amount of carbon monoxide is evolved, and with rhodium and osmium a small amount of methane is given off.

The Relation Between Hydrogenation and Dehydrogenation

From the second law of thermodynamics it follows that a catalyst cannot alter a chemical equilibrium, if the catalyst itself is unchanged; that is, in such a case the relation between the two opposing reaction velocities which form the equilibrium, is not changed by the catalyst. Therefore if such a catalyst has speeded up a dehydrogenation to a reversible equilibrium, it must as a rule also catalyze the reverse reaction, i.e., hydrogenation. Numerous cases of hydrogenation and dehydrogenation are known.[60] An exact investigation of equilibrium and reaction speeds using platinum black as the dispersed catalyzer in this case, was first carried out in Bredig's laboratory by H. G. Denham,[61] by evolving hydrogen from aqueous titanium salt solution. With constant excess of acid in aqueous solution and in the presence of platinum sponge, we have a definite equilibrium between trivalent and quadrivalent titanium salt and hydrogen. The speed with which equilibrium is established

[58] Müller, E. (with Keil, J.), *Z. Elektrochem.* 29, 395 (1923); (with Muller, F.), 30, 493 (1924); 31, 41 (1925); Paal, C. and Poethke, W., *Berichte*, 59, 511 (1926).
[59] See Ostwald, Wilh., "Lehrb. allgem. Chem.," 2nd ed., II (2), p. 498, Leipzig, 1902. van't Hoff, J. H., "Vorlesungn. ub. theor. u. physik. Chem.," 2nd ed., I, p. 211, Braunschweig, 1901. Bredig, G. in Ullmann's "Encyclopaedie d. Techn. Chem.," 5, 667 (1919).
[60] See Sabatier, *loc. cit.*; Rideal and Taylor, *loc. cit.*. Skita, A., "Katalyt. Reduction: organ. Verbindungen," Stuttgart, 1912; Bauer, R. and Wieland, H., "Reduction u. Hydrierung," Leipzig; Houben, J., "Methoden d. organ. Chemie," Leipzig; also papers of Fokin, Ipatiew, Zelinsky, Paal, Willstatter, Rosenmund, Normann, Schroeter, Armstrong, etc.; v. Braun, J. and Meigen, W., *Z. angew. Chem.*, 37, 349, 735 (1924).
[61] Denham, H. G., *Z. physik. Chem.*, 72, 641 (1910); see also Eggert, J., *Z. Elektrochem.*, 20, 370 (1914); 21, 349 (1915); Salkind, J., *Z. physik. Chem.*, 104, 177 (1923), and also other literature on velocity of hydrogenation. Regarding the rôle of Adsorption, see also Gurwitsch, L., *Z. physik. Chem.*, 107, 235 (1923).

follows both ways in certain cases, the law of a simple reaction of the first order. The effect of stirring and the small temperature coefficient (1.29 per 10°), shows that here at least, the diffusion of the substances in solution to the catalyzing platinum surface determines the reaction speed. In this work Denham emphasized the importance of surface adsorption on catalytic action.

The reversibility of hydrogenation and dehydrogenation is also demonstrated in the synthesis and decomposition of formic acid under pressure by catalysis with palladium sponge, according to S. R. Carter.[62]   J. Salkind [61] can be referred to also for work on the speed of hydrogenation of organic substances with colloidal palladium.

INORGANIC CATALYSTS AS OXIDASES AND OXIDO-REDUCTASES

When a catalyst as a "dehydrase" hastens the splitting off of hydrogen from a substance A and at the same time as a "hydrogenase" hastens the attachment of this hydrogen to a second substance B, the result thus seems to be an "oxidation" of substance A by substance B. The catalyst can therefore be described as an "oxidase" or as an "oxido-reductase."

From this point of departure G. Bredig and F. Sommer [63] first investigated the colloidal precious metals as inorganic ferment models of an "oxidase," by the "Schardinger reaction." There is an enzyme in raw milk which hastens the decolorization of methylene blue by reduction with formaldehyde. In this case it was found that electrically made colloidal sols of platinum and iridium, palladium and gold, also hasten this reduction at 70°; platinum and iridium strongly, gold and palladium faintly. At 95° palladium and gold catalyze these reactions strongly, silver sol but slightly. With all these metals and with the milk enzyme, addition of a base very markedly increases the action on Schardinger's reagent. Thus platinum sol, in a weak alkaline solution, gives the Schardinger reaction even at room temperature. If the ration between formaldehyde and the "inorganic ferment" is too large, the reaction velocity is markedly lowered. The same fact was already known for the Schardinger milk ferment reaction. The action of the "inorganic ferment" platinum can also be inhibited in the Schardinger reaction, by traces of certain "poisons" (prussic acid, mercuric chloride, iodine, hydrogen sulfide, chlorine) just as milk ferment is inhibited by free prussic acid and mercuric chloride. Here also, platinum and milk ferment do not always have the same inhibiting poisons. But this is not necessary to the analogy in question.

As Bredig and Sommer had already shown, the Schardinger reaction is a transfer of hydrogen from formaldehyde to methylene blue under the catalytic action of enzymes, and similarly under the action of "inorganic ferments." With these conceptions as a basis, H. Wieland [64] later tried to build up a theory of the oxidation processes in the organism. He studied many interesting cases of such dehydration and transfers of hydrogen from one molecule to another by catalysis with metal sponge or ferments.

The action of the colloidal metals in the place of milk ferment in the Schardinger reaction, is readily understood when we remember that the dispersed metals as "dehydrases," free hydrogen from formaldehyde. This

[62] Bredig, G. and Carter, S. R., *Ber.*, **47**, 541 (1914); *Chem. Ztg.*, **39**, 72 and 878 (1915).
[63] Bredig, G. and Sommer, F., *Z. physik. Chem.*, **70**, 34 (1909). Bredig, G., *Ber.*, **47**, 546 (1914).
[64] Wieland, H., *Ber.*, **45**, 484 and 2606 (1912); **46**, 3327, and 3339 (1913); **47**, 2085 (1914); **54**, 2353 (1921); **55**, 3639 (1922); for criticism see Warburg, O., *Biochem. Z.*, **136**, 266; **142**, 518 (1923).

hydrogen can then be transferred from the catalyst, which at the same time acts as a "hydrogenase," to the methylene blue.

These facts were brought out especially by Bredig and Sommer [63] (*loc. cit.*, p. 62) and they showed that in the reduction of methylene blue with their "inorganic ferments," the formaldehyde can be replaced even at 25° with formic acid. This reaction also possesses most of the characteristics of the Schardinger reaction—as for example, inhibition by HCN and $HgCl_2$, by heating, by excess of reducing agent, and methylene blue, etc. For this reason, indeed, Blackadder (*vide supra*) studied the catalyic evolution of hydrogen and carbon dioxide from formic acid with an eye to the existence of natural dehydrogenases.

It is known that hydrogen peroxide can reduce many substances which possess a higher oxidation potential [65] in aqueous solution, and free acid is given off. Thus hydrogen peroxide, in these instances which have been known since Thénard and Schönbein, can act as a reducing agent with evolution of free oxygen. In this way hydrogen peroxide (with the evolution of oxygen) also acts as "nascent" hydrogen; i.e., it reduces persulfuric acid and Caro's acid under the catalytic influence of colloidal platinum or palladium sponge according to T. Slater Price.[66] Similarly, according to Wieland,[66] benzoylperoxide, dehydroindigo, and sodium nitrosodisulfonate are reduced, i.e., hydrogenated by hydrogen peroxide under the catalytic stimulus of palladium black.[66] Natural ferments do not seem to work catalytically in these instances.

It was already shown by K. Ikeda,[14] W. Reinders,[14] and A. Marck,[22] that mercuric chloride under the influence of colloidal platinum, gold, and strangely enough, colloidal manganese dioxide, is quickly reduced by hydrogen peroxide to calomel, and in certain instances even to metallic mercury. This explains the following phenomena: [67] H_2O_2 reacts only very slowly at room temperature with very dilute alkaline $HgCl_2$ and likewise with very dilute colloidal gold. However, if the two are mixed, a rapid catalytic decomposition of H_2O_2 into water and oxygen takes place. The colloidal gold catalyzes the reduction of $HgCl_2$ by H_2O_2. The metallic mercury, which thus appears on the gold particles in an extremely fine state of dispersion, is, however, a very active catalyst in alkaline solution, and is here first produced from the $HgCl_2$ by the catalytic influence of the gold sol. The mercuric chloride bears a like relation to the gold sol as Pavlov's tripsinogen to enterokinase in ferment chemistry. In a similar way, R. Zsigmondy [67] used colloidal gold particles as "nuclei" or centers of growth for reduced silver particles.

The Mutase Action of Dispersed Metals

In chemistry there are many examples of so-called "disproportioning" known, in which one molecule "oxidizes" or "reduces" a second molecule of the same substance. This is a result of the willingness of the substance in question to split up into a higher and a lower state of oxidation, as for example cuprous salts split into cupric salts and metallic copper. The decomposition of hydrogen peroxide by catalase enzymes and by colloidal metals into oxygen and water, can be looked upon as such a disproportioning of H_2O_2 into a

[65] Ihle, R., *Z. physik. Chem.*, 22, 119 (1897); Bredig, G. and Muller von Berneck, *loc. cit.*, p. 292.
[66] Price, T. Slater and Denning, A. D., *Z. physik. Chem.*, 46, 103 (1903); Friend, J. A. N., *J. Chem. Soc.*, 89, 161 (1906) Wieland, H., *Ber.*, 54, 2363 (1921).
[67] Bredig, G. and Reinders, W., *loc. cit.*, 339; Bredig, G. and Weinmayer, J, Boltzmann-Festschrift, p. 841, Leipzig, 1904. Zsigmondy, R., *Z. physik. Chem.*, 56, 77 (1906).

more highly oxidized state (O_2), and into a state of lower oxidation (H_2O). It is clear from the above that H. Wieland can consider the catalytic decomposition of H_2O_2 as a "transfer of hydrogen" from one hydrogen peroxide molecule to a second one by the dehydrase and hydrogenase action of metals or catalase enzyme.[68]

We have already seen that metals as well as the Schardinger ferment, according to Bredig and Sommer, can catalytically transfer hydrogen from one molecule (such as formaldehyde or formic acid) to another (such as methylene blue) and thus effect a so-called "oxidation" of the one molecule (formaldehyde or formic acid) through the other one (methylene blue).

The disproportioning of an intermediate state of oxidation into a lower one and a higher one also occurs in the so-called Canizzaro reaction by which one molecule of an aldehyde reduces a second to alcohol, and in the reduction is itself "oxidized" to carbonic acid.

According to H. Wieland, this reaction, as for example with salicyl aldehyde, is speeded up by means of a milk ferment "mutase." Wieland [69] also touches upon mutase of milk as a catalyst which transfers hydrogen from one hydrogenated aldehyde molecule to a second aldehyde molecule, and which is probably identical with the Schardinger enzyme. This Schardinger milk mutase or "dehydrase" would carry hydrogen between two molecules of salicyl aldehyde just as, for example, colloidal platinum (according to Bredig and Sommer) transfers hydrogen between formaldehyde or formic acid, and methylene blue. Relative to this, Müller [70] showed that formaldehyde in aqueous solution can also be catalytically disproportioned by platinum metals, splitting, according to the nature of the catalyst and the composition of the solution, into formic acid and hydrogen, and finally into methyl alcohol and carbonic acid. The higher the degree of dispersion of the metallic catalyst, the more easily the "fermentation" of formaldehyde by "inorganic ferments" takes place.

This transfer of hydrogen can also go on within the -ame molecule from one atom of carbon to the other. This seems to be the case in the decomposition of pyrotartaric acid into acetaldehyde and carbon dioxide. $CH_3 \cdot COCOOH = CH_3 \cdot CHO + CO_2$. According to C. Neuberg,[71] the reaction is catalyzed by an enzyme "carboxylase," and according to E. Müller and F. Müller,[71] it can be catalyzed by highly dispersed metallic platinum.

Here again we see the striking analogy between enzymes (namely the catalyses, dehydrases, oxydases, mutases, carboxylases) and highly dispersed metals.

IRON-CONTAINING FERMENT MODELS

According to the theory of H. Wieland referred to above, oxidation processes arise by catalysts or oxidizing ferments behaving as dehydrases, or hydrogenases, "activating" the hydrogen or oxidizable substances, and thereby enabling it to split off and become attached to "hydrogen receptors"—as for example, molecular oxygen, oxygen-containing (quinone) or unsaturated molecules which do not contain oxygen (methylene blue). On the other hand O. Warburg [72] has recently offered a different theory of respiration ferments,

[68] Wieland, H., *Ber.*, **55**, 3647 (1922).
[69] Wieland, H., *Ber.*, **47**, 2089 (1914).
[70] Muller, E., *Z. Elektrochem.*, **27**, 558 (1921); **31**, 41 (1925).
[71] Neuberg, C., *Ber.*, **54**, 3626 (1922). Muller, E., *Z. Elektrochem.*, **31**, 45 (1925).
[72] Warburg, O., *Ber.*, **58**, 1001 (1925) Millon, M. E., *Compt. rend. (Paris)*, **19**, 270 (1844). Warburg, O., "Ueber die Katalyschen Wirkungen der lebendigen Substanz," Berlin, 1928.

according to which iron behaves as an oxygen carrier. The iron, according to Warburg, acts in its bivalent form with the molecular oxygen which disappears in respiration. This more highly oxidized iron, then oxidizes the organic material as it reverts back to the ferrous state. The iron here plays the rôle of a catalytic oxygen carrier.

It is well known that iron is essential to the living cell. To back up his conception, Warburg (with O. Meyerhoff) showed that the oxygen consumption of unfertilized sea-urchin eggs rises according as the iron content of the cell substance is increased. It has been known for a long time (Claude Bernard) that minimal amounts of prussic acid (1/1000–1/100,000 normal solution) stop or inhibit the respiration of the cell. The same is known of hydrogen sulfide and arsenic acid. Warburg explains the poisonous action of these substances on respiration by saying that they react chemically with the iron in the organism and thus make it inactive as a catalyst. Further, to support his theory, Warburg constructed iron-containing catalyst models of respiration ferments, which because of the development of a large amount of surface, are dispersoids whose importance Bredig had recognized for years.[14] Warburg showed that blood charcoal or "hemin charcoal," i.e., charcoal made by heating hemin to red heat, can oxidize catalytically, with molecular oxygen at low temperature, a number of organic substances in aqueous solution, such as oxalic acid, amino acids, i.e., leucin, cystin, etc. Oxalic acid in aqueous solution, leucin, cystin, etc., are oxidized with the evolution of carbon dioxide under the catalytic influence of blood charcoal or hemin charcoal suspended therein at 38° C., by shaking in the presence of atmospheric oxygen.

According to H. Freundlich,[73] the time law governing this catalysis involves the adsorption isotherm of oxalic acid at the charcoal interface. We must recall that the adsorption isotherm held good, according to Blackadder,[56] in the time law of the decomposition of formic acid by dispersed rhodium. O Warburg [74] has further shown that there is an inhibition of the oxidation of oxalic acid on the blood charcoal by readily adsorbed "capillary-active" substances like the narcotic urethane, according to Traube's law, and in approximate proportion to the amount of this substance adsorbed; the inhibition being similar to that produced on the oxidation velocity in the respiration of red blood cells.

We must recall (*vide supra*) that according to Meyerhoff [51] the catalase action of colloidal platinum on H_2O_2 can be likewise diminished by such capillary-active substances. The same phenomena were demonstrated in the catalytic oxidation of cystin. The explanation is that the oxidizable organic substance is crowded away from the surface of the catalyst by these strongly adsorbed narcotics. Bredig and Müller von Berneck [14] (*loc. cit.*, p. 339) had already remarked on the possibility of this explanation. At any rate, these researches of Warburg and others are important in that they show that oxidation on blood charcoal and also in the cell, proceeds catalytically on the surfaces of very highly dispersed particles.

That the specific catalytic transfer of oxygen is here attributable to iron, is shown by O. Warburg, by the fact that the respiration of the cell, as is also the case in the oxidation of oxalic acid and formic acid in his charcoal models, is dependent upon a certain iron content, even though it be a small one.

[73] See also the splendid exposition by Freundlich, H., "Kapillarchemie," 3rd ed., p. 309, 1923; Freundlich, H. and Bjercke, Z. *physik. Chem.*, 91, 1 (1916).
[74] Warburg, O., *Archiv. Physiol.*, 155, 547 (1914); Z *Elektrochem.*, 28, 70 (1922); *Naturwissenschaften*, 13, 992 (1925); Rideal, E. K. and Wright, W. M., *J. Chem. Soc.*, 3182 (1926).

"Charcoals of the catalytic activity of hemin charcoal arise only when two conditions are fulfilled: when the initial material contains non-volatile nitrogen, and in addition, iron. If certain nitrogenous substances are purified so that they contain but little iron, and are calcined, the result is a faintly active charcoal. If these charcoals are soaked with an iron salt and heated red-hot, they become activated, and acquire the catalytic activity of hemin charcoal. The richer in iron, the more active is the nitrogen containing charcoal, until a maximum activity is reached with an iron content of a tenth of a milligram per gram of charcoal. No activity developed when nitrogen con-
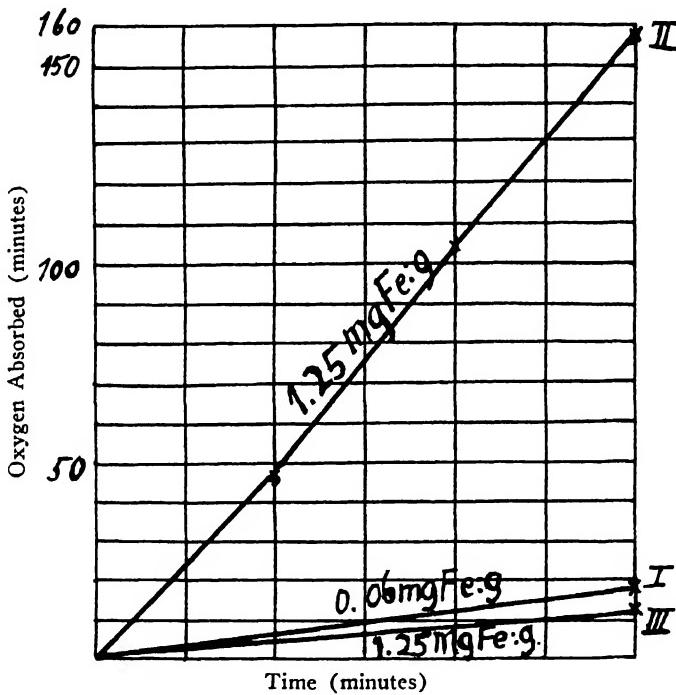
FIG. 7.

taining carbon was brought to red heat with other metals, or when substances free from nitrogen were heated red-hot with iron. Thus the catalytically active substance of hemin charcoal is iron, but not iron in any form whatever, but iron bound to nitrogen."

Furthermore, according to Warburg, the catalytic action of the iron-nitrogen complex is specifically inhibited by prussic acid, as in the respiration of the cell. 1/10,000 normal prussic acid markedly decreased the activity of hemin charcoal and all the impure iron compounds which were tested, as Figure 7 shows. Curve I shows the transfer of oxygen to leucin by charcoal poor in iron; curve II, the same charcoal after its iron content had been increased. Curve III shows the oxygen transfer by charcoal rich in iron in a 1/1000 normal prussic acid solution. It is plainly seen that the activity of the charcoal increases as its iron content, and is markedly inhibited by prussic acid.

The work of Mathews and Walker [75] on the inhibiting influence of very small amounts of prussic acid on the oxidation of cystein solution with air, finds its explanation, according to Warburg, in the fact that the very slow oxidation of cystein by air was considerably speeded up by even 1/100,000 mg. of iron in 10 cc. solution, and was naturally inhibited by prussic acid. According to Harrison,[75] cystein-glutaminic acid behaves likewise. In all these cases prussic acid has the same "poisonous" effect on the catalytic properties of iron, as in the earlier work of Bredig on the heavy metal catalysis of the decomposition of hydrogen peroxide (*vide supra*).

According to work of O. Warburg and Sh. Toda,[72] traces of iron also play an important catalytic rôle in the oxidation of oxalic acid with hydriodic acid. The inhibition of the reaction by a minimal amount of prussic acid observed by M. E. Millon,[72] can be traced to the decrease in the catalytic activity of the iron. This "poisoning" of the iron catalyst can be counteracted, according to Warburg, by blowing in a stream of air, just as in the earlier work of Bredig,[14] Berneck, Ikeda, and Senter [23] already described above, where platinum and hemase catalase were "restored" from "poisoning" with prussic acid. H. Blaschko, and Euler and Josephson [23] have made the same observation. In all these cases the "poisoning" of the catalysts with prussic acid is reversible. If the prussic acid is chemically bound to the catalyst, it must either be an unstable compound or else one that is easily oxidized.

In view of the catalytic relations in the cell and ferment respiration as in his organic carbon-iron models, Warburg expresses the following views:

"All the phenomena connected with the respiration of living substances can be traced back to the action of two kinds of forces; to the action of nonspecific surface forces, and to the action of specific chemical forces.

The non-specific surface forces concentrate the substances which oxidize, on the surface of the solid constituents of the cell, and these surfaces, not the cell fluids, are the seat of the oxidation processes. Respiration is a surface reaction, and as such is inhibited by all substances which, without being oxidized themselves, crowd the material to be oxidized away from the surface. This is the explanation for the action of the chemically indifferent narcotics, which bring the processes in the cell to a standstill by crowding other substances away from the surface. Easy as it is to follow out the existence and action of the non-specific surface forces in the living cell, so certain is it, on the other hand, that these forces are not sufficient to carry out a reaction between the organic substances and oxygen. A reaction only takes place when chemical forces are present in addition to the surface forces. The adsorbing surfaces in the cell are not homogeneous, but contain a substance which has a strong chemical attraction for oxygen. This substance is the respiration-ferment."

Twenty-five years ago in their work on the "inorganic ferments," i.e., on inorganic models for catalase ferment and others, G. Bredig and Müller von Berneck [14] (*loc. cit.,* p. 341) stated the following observations:

"Thus we see that even on such large amounts of surface as we have in colloidal solutions of gelatin, ferric hydrate, silica, and precipitated alumina, no noticeable catalysis of H_2O_2 took place. Therefore the catalysis of hydro-

[75] Matthews, A. P. and Walker, S., *J. Biol. Chem.,* **6,** 29 (1909). See also Meyerhof, O., *Archiv. Physiol.,* **200,** 1 (1923); *Biochem. Z.,* **150,** 1 (1924). Harrison, D. C., *Biochem. J.,* **18,** 1009 (1924) on the rôle of Manganese in Oxydase-Reactions after Bertrand and Dony-Hénault, see *Bull. Acad. roy. Belgique,* 1908, 105, 1909, 342, and also Rideal and Taylor, *loc. cit.,* p. 349. Hennichs, S., *Berichte,* **59,** 218 (1926); *Biochem. Z.,* **17,** 314 (1926). Warburg, O., *Berichte,* **59,** 739 (1926). Willstätter, R., *Berichte,* **59,** 1871 (1926).

gen peroxide is dependent upon a specific sort of surface such as is found in platinum."

Elsewhere Bredig [15] (1901, *loc. cit.,* p. 98) remarks:

"All these processes have the significance of a clear-cut analogy between the contact reactions in the inorganic world and the ferment reactions in the organic world. My colloidal catalysts involve reactions which take place on enormously developed surfaces. Therefore it is probable that such is the case with the reactions of ferments, enzymes, blood corpuscles, and oxidizing and catalyzing organic material. Thus we see that the organism develops its large amount of free surface in the tissues, and colloidal ferments, not only because osmotic processes are needed, but also because of the great catalytic activity of such surfaces. If Boltzmann says that the struggle of living things for existence is a struggle for free energy, then, surely, free surface energy is one of the most important kinds of all for the organism."

"In closing, it is scarcely necessary for me to say that the metals and the enzymes are not identical. However, when the surprisingly numerous analogies are considered, colloidal metal sols (and probably also MnO_2 sols, etc.) must at least be thought of in many instances as inorganic models of organic enzymes because they possess the following properties:

(1) strong catalytic potency;

(2) a colloidal, very often labile state, with enormous development of free surfaces which may often suffer irreversible changes;

(3) ability to bind chemically, certain substances by building up intermediate compounds or by adsorption. (Also semipermeability for certain substances may perhaps be included here.)

At any rate, these researches will prove to physiologists the oft repeated phrase of Wilhelm Ostwald, that thorough investigation of the laws of chemical reaction velocity and of catalysis is much to be desired."

Whoever surveys the ferment literature of the last twenty-five years, will again find many of these same points which formerly were not sufficiently emphasized.[76]

[76] Among the great mass of literature on *catalysis,* the following reviews may be mentioned: Ostwald, Wilh., "Ueber Katalyse," Ostwald's Klassiker, Nr. 200 (Leipzig, 1923; Langmuir, I., *Trans. Farad. Soc.,* 17, 607, 621 (1922); Abel, E., *Z. Elektrochem.,* 19, 933 (1913); Bancroft, W. D., *Trans. Am. Elektrochem. Soc.,* 37, 21 (1920), *J. Physic. Chem.,* 21, 573, 644, 734 (1917), 22, 22 (1918), 27, 801 (1923); *J. Ind. Eng. Chem.,* 14, 326, 444, 545, 642 (1922). Falk, K. G., "Catalytic Action," New York, 1922; Rideal, E. K. and Taylor, H. S., "Catalysis in Theory and Practice," London, 1926; Taylor, H. S., *J. Phys. Chem.,* 28, 897 (1924); 30, 145 (1926). Rideal, E. K., *Chem. Ind.,* 42, 614 (1923); Bredig, G., "Katalyse," in Ullmann's "Techn. Encyclopaedie," 5, 665 (1919); Hinshelwood, C. M., "Kinetics of Chemical Changes in Gaseous Systems," Oxford, 1926; Huckel, W., "Katalyse mit Kolloiden Metalle," Leipzig, 1927; Willstätter, R., *J. Chem. Soc.,* 1359 (1927); Mittasch, A., *Berichte,* 59, 13 (1926).

Enzyme Action in Relation to Surface Catalysis

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Enzymes Are Catalysts

The jest is sometimes made that when the biologist encounters a phenomenon which he cannot understand, he creates an enzyme. Superficially there is justice in the thrust, for physiological literature is strewn with the hasty use of the name as an encompassing explanation of random observation. When, however, we consider what physiology implies in the use of the term, it may be accepted as a form of description in every way as legitimate as are the shibboleths of the physical sciences.

"Enzymes are catalysts produced by the living cell." The definition permits us to label as an enzyme any material of biological origin exhibiting catalytic activity, provided there be absent those characteristics of life which are broadly defined by the capacity for growth and reproduction. The use of the term indicates that a measure of success has been attained in the chemical analysis of the vital activities of the cell. It indicates nothing more. Such lack of discrimination may be unfortunate, yet it is probably wise, since we know so little of the nature of enzymes that we can ill-afford to be exclusive. It is indeed doubtful if it be wise to qualify the simple definition, as is often done, by attributing to enzymes in general the special characteristics of thermolability or colloidal character. The student of enzyme action is confronted at every turn by the elusive nature of his problem. An enzyme is recognisable only by its activity. There are no independent criteria for its identification. The best that he can do is to seek to establish relations between the activity of an enzyme and the physical and chemical characteristics of the material with which the activity is associated in the hope that he may approach the problems of what an enzyme is and how it works.

It is not within the province of the present article to labour the argument that enzymes are catalysts. Full discussion will be found elsewhere.[1, 2, 3, 4] Suffice to say that, in a general sense, enzymes comply with the requirements of true catalysts in respect of their effects upon velocities of reaction and upon equilibria and of the absence of a stochiometric relation to the reaction catalysed. It is true that certain important discrepancies have from time to time been demonstrated, but these continue to submit to explanation as the

[1] Bayliss, Sir W. M., "The Nature of Enzyme Action," Monographs on Biochemistry, London, 1919.
[2] Falk, K. G., "The Chemistry of Enzyme Action," New York, 1921.
[3] Waksman, S. A. and Davison, W. C., "Enzymes," New York, 1926.
[4] Euler, H., "Chemie der Enzyme," Munich, 1925.
The above monographs or text-books may be consulted for general discussions of the nature of enzyme action and for bibliographies of original papers.

special conditions of enzyme activity become more fully appreciated. In particular, it has been the recognition of the fact that enzymes are, or are intimately associated with, matter in the colloidal state and that the behaviour of enzymes must be limited by the characteristics peculiar to heterogeneous systems, which has directed and enlightened both the analytical and kinetic studies of enzyme action. Berzelius, to whom we owe the word "catalysis," perceived in the fermentation of sugar by yeast "an indwelling force whose nature is yet unknown" allied to the decomposition of hydrogen peroxide by solid bodies such as platinum, silver and fibrin. Whether such an analogy would have been made had the knowledge been then available that sugar fermentation could be reproduced in yeast juice in which the microscope betrayed no structural elements may be questioned, for this discovery of Büchner made its most obvious contribution to biology in its emphasis upon the important part played in the vital economy by the "unorganised ferments" of the protoplasm. It is, therefore, a piquant reflection that we are, today, returning to the suggestion of Berzelius when we associate the peculiarities of enzyme action with the colloidal character of the systems concerned.

COLLOIDAL CHARACTER OF ENZYMES

It is a matter of wide experience that enzymes fail to dialyse, or pass only very slowly, through parchment and similar membranes. They do not diffuse at measurable rates into gels. Their solutions exhibit the phenomenon of cataphoresis indicating that the catalytic activity is associated with an electrically charged phase. They are irreversibly inactivated by heat and their behavior towards such solvents as alcohol and acetone is reminiscent of typical hydrophylic systems. High concentrations of electrolytes generally cause visible coagulation, but the activity of the solutions is notably affected by concentrations of salts much below those necessary to so aggregate the colloidal material as to determine actual flocculation. An immense literature has grown round these varied reversible and irreversible effects, designed to emphasise the colloidal character of enzymes, to establish optimum conditions for their activity and to achieve some analysis of the enzyme complex. Indeed, it is upon them that differential methods of separation and purification have been elaborated.

The student will find this a bewildering literature. He will have to attempt to disentangle such factors as the electrical charge on the enzyme phase, the effect of the hydrogen ion activity and such ionic effects as those represented by Hardy's Rule and the Hofmeister Series. At the same time he must distinguish individual phenomena of the type of catalytic poisoning and co-enzyme and anti-enzyme effects. The elusive character of our definition is here a serious impediment in collating the work of different observers, since enzyme activity seems to be so sensitive to conditions inadequately within experimental control that it is, in general, only possible to obtain comparable data within the limitations of a single technique. As Sörensen has pointed out, the time factor is of supreme importance in the study of the activity and the inactivation of enzymes. Thus temperature, hydrogen ion activity and time are so interrelated that the determination of the optimum temperature or pH is without significance if the time of submission to such conditions be not defined. When we appreciate that the optimum temperature is, in all probability, itself a balance between the temperature coefficient of the reaction catalysed and that of the inactivation of the enzyme by heat—which, like the

temperature coefficient of the heat coagulation of proteins, is probably high—the significance is clear. The effects of hydrogen ion concentration and of electrolytes in general are likely to be complex equilibria of a similar type and will be, equally, a function of the time of observation.

Although we would avoid a sterile discussion of the effects of electrolytes on enzyme activity, some attention is due to the fundamental importance of hydrogen ion activity to enzymes. The occurrence of a relatively narrow range of hydrogen ion concentration within which an enzyme is active and the relation of the activity to the pH within that range, together with the knowledge that the enzyme phase is electrically charged has naturally led to the suggestion that the enzyme is itself an ionisable structure and that the degree of its dissociation determines the extent of its activity. Thus it has been suggested that invertase [4] and catalase are active as undissociated molecules, that only the cations of pepsin and amylase [5] are active and that trypsin,[5] lipase and maltase are active catalysts when negatively charged.[6] The similarity between the activity — pH curve of many enzymes and the acid-base titration curve of an ampholyte, is tempting graphical evidence for the view that enzymes are amphoteric. This is not, however, necessary to explain the hydrogen ion optimum provided the degree of dissociation of the substance undergoing change also governs the rate of the reaction. Widmark [7] has offered a simple model of a system of this type. Aniline catalyses the ketone hydrolysis of acetoacetic acid and the reaction has a definite optimum rate at pH 4.08. From a knowledge of the ionisation constants of aniline and of acetoacetic acid it can be shown that this optimum and the relation of the rate of the reaction to the hydrogen ion concentration, would necessarily follow from the assumption that it was the undissociated molecule of aniline which catalysed the hydrolysis of the undissociated molecule of the acid. In the case of enzymes the studies of Dernby,[8] Northrop [5] and Levene and Simms [9] on the relation of certain proteolytic enzymes to the dissociation constants of peptides and proteins on which they act represent interesting attempts to further this argument. It must not be forgotten, however, that enzymes exist in aqueous solution as a separate phase and there must remain some uncertainty whether the electric charge originates in the ionisation of a specific grouping in the surface or whether it should rather be attributed to a preferential adsorption of ions.

KINETICS OF ENZYME ACTIVITY. SURFACE CATALYSIS

When we turn to the detailed kinetic study of enzyme activity we are again confronted by a bewildering literature. By restricting consideration to the more careful and exhaustive investigations, however, we shall be able to reconcile many of the apparent anomalies with the heterogeneous character of enzyme systems. It is a familiar fact that in the catalysis of the hydrolysis of *e.g.* esters in homogeneous solution, if allowance be made for autocatalytic effects, the monomolecular rule has been established. Moreover the velocity is proportional to the concentration of the catalyst. When the appropriate enzyme is substituted these considerations no longer hold. In the majority of cases the velocity constant calculated for a monomolecular reaction steadily

[5] Northrup, J. H., *J. Gen. Physiol.*, papers in vols. 3-7 (1919-1925).
[6] Michaelis, L. and Rona, P., *Biochem. Z.*, 57, 70 and 148, 60, 62 (1913).
[7] Widmark, E. and Jeppsson, C. A., *Skand. Arch. Physiol.*, 240, 43 (1922).
[8] Dernby, K. G., *Biochem. Z.*, 81 (1917).
[9] Levene, P. A., Simms, H. S. and Pfaltz, M. H., *J. Biol. Chem.*, 61, 445 (1924).

. falls as the reaction proceeds. With low concentrations of enzyme the velocity of the reaction is proportional to the concentration of enzyme, but, as the latter is increased, an exponential relation is betrayed and a point is reached, at fairly low concentrations of enzyme, beyond which further increases are without effect upon the velocity.

Bayliss[1] was the first to point out the similarity between this picture and that represented by the adsorption isotherm of Freundlich, and it is due largely to his appreciation of the significance of adsorption to the process of enzyme catalysis that we owe the clarification of the many kinetic studies which have been made. In support of his contention that the conditions governing adsorption were those which controlled the activity of the enzyme Bayliss was able to elaborate a powerful argument. In the first place it was demonstrated that enzymes would exhibit definite activity when aggregated to such an extent that they could be filtered from solution and could, therefore, be described as insoluble. In a number of cases it has been shown that experimentally demonstrable adsorption complexes between enzymes and their substrates are formed. Moreover, the effects of surface active substances, such as a series of alcohols, upon the activity of an enzyme have been shown to be in the order in which they reduced the surface tension at a water interface, and presumably, therefore, in the order in which they would tend to displace the substrate from the surface of the catalyst.

In more recent studies, later to be discussed, this argument has been notably supported.

The reader will perceive that he has been led to the point at which the suggestion must be made that it is by a comparison of surface catalysis and of enzyme activity that enlightenment must be sought on the mechanism of the latter. We are entitled to distinguish four phases in a surface catalysed reaction :

1. The rate of diffusion of the reactants to the surface.
2. The rate of adsorption at the surface.
3. The rate of the chemical reaction at the surface.
4. The rate of removal (evaporation) of the products from the surface.

In an enzyme solution the catalytic phase is so finely dispersed that the diffusion factor can, almost certainly, be neglected, as also may the rapid process of adsorption. It is probable, therefore, that the velocity of reaction which is actually observed will be that of the chemical reaction upon the surface, except in so far as it may be continuously modified by diminution of active surface through progressive occupation by the products of the reaction. That an enzyme does adsorb the products of the reaction catalysed may in several cases be actually demonstrated, and, indeed, if the relation of adsorption to activity be accepted, is implicit in the thesis that an enzyme catalyses both reactions of a reversible change.

The number of examples of synthesis by means of enzymes continues to increase and to further emphasise this contention. The extensive studies of the action of pepsin and trypsin by Northrop[5] have been successful, indeed, in giving quantitative expression to the effects of products of the reaction upon the velocity observed. In the case of these enzymes the divergence of the velocity curve from that characteristic of a monomolecular reaction is notable and cannot, as Bayliss showed, be explained by an irreversible inactivation of the enzyme. It can not, therefore, be attributed to the irreversible

diminution of active surface by aggregation. The concept of the surface being immobilised by the progressive occupation of the surface by the products of the reaction provides, on the other hand, a reversible inactivation actually amenable to experimental measurement. In spite of the complications involved in the colloidal character of the reactant, Northrop has been able to show that combination of pepsin or trypsin with products of the hydrolysis of the protein quantitatively explains the peculiarities of the velocity curve.

Another instructive case is that of the reversible inactivation of the hydrolysis of cane sugar by invertase by the products of the reaction.[10] That this effect represents something more than the operation of the Mass Law for simple reversible systems, is indicated by the fact that laevulose is much more potent an inhibitor than is dextrose. More recently a particularly interesting case of the same phenomenon, from the group of oxidation-reduction enzymes, has been studied in some detail.[11] There occurs in milk an enzyme—called the xanthine oxidase—which is able to effect the oxidation of xanthine and hypo-xanthine in the presence of either oxygen or a number of "hydrogen acceptors" such as methylene blue. It has been found that this activity is inhibited not only by the products of the reaction, e.g., uric acid, but also by excessive concentrations of the purine reactant itself. To explain this it would seem to be necessary to picture an adsorption of both purine and methylene blue in such fashion that excess of the former appropriates the available surface to the exclusion of the dye, with the result that the catalytic acceleration of the reaction is no longer possible. The case of a reactant impeding its own reaction in a surface process is not an unusual phenomenon as Langmuir has shown in respect of a number of gaseous reactions at low pressures at metallic surfaces, so that this example from the field of enzyme chemistry will give us further confidence to compare catalysis at metal surfaces and in enzyme systems.

The familiar Rule of Schultz and Borissov that the velocity of an enzyme action is proportional to the square root of the concentration of enzyme is an empirical relation which is often quoted, though experience teaches that it holds only under restricted experimental conditions. Langmuir [12] has pointed out that by integration this rule may be expressed by the statement that the velocity is proportional to the concentration of enzyme, inversely proportional to the concentration of the products of the reaction and independent of the concentration of the reactants. He shows how this condition would be achieved, assuming surface catalysis to be determined by the formation of an orientated monomolecular film, if the products were adsorbed to such an extent as nearly to cover the active surface.

At the risk of digression, reference may be made to an interesting case of irreversible inactivation by the products of an enzyme reaction, which may be contrasted with the reversible effects discussed above.[11] When oxygen acts as the hydrogen acceptor for xanthine oxidase, the progress of the reaction indicates a progressive destruction of the enzyme. This is not found when methylene blue is employed as the oxidising agent with the result that the velocity curves in the two cases are quite different. It has been found that the cause of the difference is the production of hydrogen peroxide by the reduction of the oxygen. When precautions are taken to remove this as

[10] Michaelis, L. and Menten. M. L , *Biochem. Z*, **49**, 339 (1913).

[11] Dixon, M. and Thurlow, S , *Biochem. J.*, **18**, 976 (1924); Thurlow, S., *Biochem. J.*, **19**, 175 (1925).

[12] Langmuir, I., *J. Am. Chem. Soc.*, papers in vols. 38-40 (1916-8).

rapidly as it is formed the anomaly in the velocity curve disappears, clearly demonstrating that hydrogen peroxide has a toxic action on the enzyme, leading to a progressive diminution in active mass of catalyst as the reaction proceeds.

SURFACE "ACTIVATION"

Our problem is by no means solved by the acknowledgment that the forces which determine adsorption are the forces which govern the activity of an enzyme. We have still to consider why adsorption at a surface should result in an acceleration of the reaction concerned. Bayliss was inclined to the belief, following the early suggestion of Faraday respecting the catalytic activity of metallic surfaces, that the increased active mass at the surface may be sufficient to explain the increased velocity observed. At the same time it was acknowledged, as again Faraday foresaw, that in the process of adsorption there may well result molecular stresses and distortions such as to predispose the reacting molecule to change. That is to say, the possibility for what we are now accustomed to call "activation" was conceded. Some such qualification must, indeed, be made. We have to explain why it has been possible to demonstrate the adsorption of reactants under conditions in which no reaction followed, and why adsorption to a surface of two substances capable of interaction does not necessarily lead to accelerated reaction.

It has been customary in discussions on the nature of enzyme action to distinguish the physical, i.e., the adsorption theory of enzyme action from the chemical theory. The latter, following conventional theories of catalysis in homogeneous systems, postulates the formation of intermediate compounds of the enzyme and one or all of the reactants such that the compound readily breaks down into the free enzyme and the products of the reaction. The critical requirement of this theory is that the velocities of the consecutive reactions

$$\text{Enzyme} + A \rightarrow \text{Enzyme} - A \rightarrow \text{Enzyme} + B + C$$

should each be greater than that of the straight-forward reaction

$$A \rightarrow B + C$$
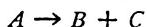
As applied to enzyme reactions this has failed to prove a profitable hypothesis, for the simple reason that it has not been possible to isolate the hypothetical intermediate product and examine its behavior. It demands, moreover, definite ideas of the chemical nature of the enzyme itself—and of this we have scarcely begun to learn. The adsorption theory, on the other hand, does, as we have seen, permit a useful interpretation of the main catalytic activities of enzymes and modern views of the nature of adsorption go far towards reconciling the physical theory with our natural prejudice for finding a chemical explanation for catalytic processes which appear on their face to be chemical phenomena.

The forces which determine adsorption, at least in monomolecular layers, are undoubtedly chemical in nature, being simply the residual electromagnetic field of the atoms occupying the surface. Surface energy becomes, therefore, an expression of the primary or residual valency forces of the surface atoms, and there is no reason why an adsorption complex should not reflect as definitely as does a stochiometric compound, the chemical characteristics of the atoms entering into the combination. Adsorption *per se* will not determine a catalytic effect. The nature of the complex formed at the surface will

decide whether or not reaction to form the postulated products will be accelerated. Stated in this way the physical theory becomes indistinguishable from the chemical theory in substance, though there is a difference in language. Perhaps, after all, the real problem is that of finding the most useful definition of what we call the active state. The organic chemist sees a process of activation in the formation of an intermediate compound between enzyme and reactant which is more reactive than is the uncombined reactant. He would define an active molecule by means of a definite peculiarity of chemical structure. The physical chemist, on the other hand, opposes a dynamic to a static view and is content to picture an active molecule merely as one in a higher quantum state than that characteristic of the average body of molecules, and will compromise with the structural view only so far as to imagine a distortion of the electronic organisation of the molecules. The advantage of the latter view is that it permits of an activation which may fall short of complete reorganisation of the molecule into a new and static chemical structure. In the main, thermodynamics and structural chemistry have alike dealt with molecules in the average. It becomes increasingly probable that chemical reaction and catalysis in particular are concerned with the egregious rather than the average molecule.

The immediate difficulty is to find some experimental method for the identification and study of the active state other than inference from the kinetics of change. It is of some interest, therefore, that Cannan, Clark and Cohen [13] believe that they have observed the process of activation in the case of certain oxidation-reduction enzymes under conditions in which no chemical reaction was in progress. They found that substances which were capable of oxidation or reduction when these enzymes were present, but not when they were absent, also developed reproducible potentials at an electrode in the presence but not in the absence of the enzyme. They suggest that in these cases "activation" may, perhaps, be identified with "electromotive activation."

The significant thing is that the latter can be observed under conditions in which no chemical reaction is in progress because of the absence of the necessary oxidising or reducing agent. This is clearly an attempt to replace a strictly structural concept—Wieland's theory of the activation of hydrogen atoms—by a less rigid picture of a molecule in which the electron restraints are to some degree loosened. That this has assumed electrochemical language is natural, but it is significant that Quastel [13a] has independently and from a kinetic standpoint arrived at a similar electrochemical theory of the "activations" effected by bacterial reductases. The latter theory, moreover, represents an interesting attempt to exploit modern views of the mechanism of surface catalysis particularly in respect of the localisation of activity in "active spots" and the significance of the specificity of enzymes. To these questions we may now turn.

CHEMICAL NATURE OF ENZYMES IN RELATION TO SURFACE

Of recent years one of the most effective methods of investigating surface reactions has been by studies of the reversible poisoning of the surface. The same method is finding application to enzymes. The reader will recollect, indeed, the plausible parallel drawn by Bredig between the poisoning of his

[13] Cannan, R. K., Cohen, B. and Clark, W. M., *U. S. Public Health Reps.* 1926, Suppl. 55.
[13a] Quastel T. H, *Biochem. J.*, **20**, 166 (1926); **21**, 1225 (1927)

metallic sols—he called them "inorganic ferments"—and the poisoning of enzymes.*

Since Faraday first noted the reversible inactivation of platinum by such gases as hydrogen sulfide and hydrocyanic acid, the formation of a film of the poison on the surface has been called in as explanation. The work of Lord Rayleigh, Hardy and Langmuir led to our appreciation of the fundamental importance of the monomolecular film and the more recent extension of these views by Rideal, H. S. Taylor and others have provided a conception of surface catalysis the significance of which to the study of enzyme action cannot be overlooked. We have learnt to look on the catalytic surface no longer as a uniform area, but rather as a relatively inactive surface upon which are found restricted areas of intense catalytic activity. Moreover there is a high degree of specificity in these areas, so that one surface may be able to catalyse a variety of distinct reactions at spacially separate portions of its surface. It follows, therefore, that the total adsorbing capacity of the surface may not necessarily parallel its catalytic activity towards any particular reaction, nor need the suppression of these properties by poisons run parallel. Although this has complicated the analysis of the activity of a surface, some success has already been achieved in determining the ratio of total surface to the surface effective in catalysing a particular reaction, and in relating this active area to its peculiar chemical and physical characteristics. These are conclusions of real moment to enzyme chemistry, for there is increasing evidence to suggest that the catalytic activity of enzymes is restricted to certain areas of the surface of the enzyme phase. This is no new idea. Long ago Bertrand regarded enzymes as consisting of two components. One was a simple atom or group, possibly inorganic, having limiting catalytic activity in itself. The other was a colloidal material whose function was to accentuate and render specific—to "support"—the activity of the catalytic component. An enzyme was thus comparable to surface catalysts of the type of platinised asbestos and stabilised metallic sols.

Willstätter, reviewing his laborious attempts to isolate enzymes in the pure state, has recently added his authority to this conception of the nature of enzymes.[14] An enzyme, he considers, consists of an active catalyst and an inert colloidal carrier. The latter is not specific and may, in part, be separated and replaced by other suitable colloidal material. On the other hand, it is not possible to obtain an active enzyme in the form of the carrier-free catalyst as the latter appears to be inherently unstable in the absence of protection. This conforms to the familiar observation of biochemists that the further the purification of an enzyme is carried the more labile it becomes to mild experimental conditions.

There has long been good reason to suspect a relation between iron or manganese and mechanisms involved in the respiration of living cells—i.e., in what is known as the activation of oxygen. One of these elements is constantly found associated with most oxidase systems even after extensive purification,[15] and Warburg[16] has developed a plausible argument for the view that iron is essential to the utilisation of oxygen by cells. In co-operation with Meyerhof he has shown that the consumption of oxygen by living cells in

* See papers by Bredig and Willstätter in this volume; also papers by Hardy and Langmuir in Vol. I of this series. *J. A.*

[14] Willstätter, R., *J. Chem. Soc.*, 1927.

[15] Willstätter, R. and Stoll, A., *Liebig's Ann.*, 416, 21 (1918).

[16] See "Chemical Dynamics of Life Phenomena," O. Meyerhof, New York, 1923.

vitro occurs upon the surface of the cells. It is a surface process. Further, it is a process depending upon the presence in the surface of catalytic iron. In this study the behavior of hydrocyanic acid as a specific poison for autoxidations catalysed by the heavy metals, has proved of great value. Hydrocyanic acid will, in low concentrations, inhibit the respiration of cells and the activity of catalase and many oxidases and a relation has been established between the toxic concentration and the amount of iron present in the system. Readers will be familiar with Warburg's ingenious employment of a model of biological oxidations in the form of the catalytic activity of charcoal in the oxidation of substances of biological interest. He has shown that this depends upon the presence in the charcoal of active areas containing iron, has established the poisoning effect of hydrocyanic acid on these areas and has shown that the chemical character of the iron present is also important. The most active charcoal studied, for instance, was one prepared from haematin which is the iron-containing portion of the haemoglobin molecule.

In view of the conclusion which is suggested by the work of Baudisch,[17] Conant,[18] Keilin[19] and others that haemoglobin is nearly related to the forms of iron which are assumed to control the activity of the oxidative enzymes, it is highly interesting that catalytic activity is found in some degree in the molecular skeleton which is preserved in the haematin charcoal. The experiments of Baudisch are of particular interest and have not yet received the attention they deserve from biologists. He has shown that ferrous hydroxide and bicarbonate and also preparations of magnetite give those color tests usually associated with peroxidases, and that they catalyse the oxidation of such substances as the purines and lactic acid by oxygen. The activity of the hydroxide and bicarbonate is dependent, however, upon the amorphous character of the precipitate and is lost as ageing proceeds. Magnetite may be oxidised under controlled conditions to a product chemically indistinguishable from the higher oxide, haematite. Its catalytic and magnetic properties are, however, retained, as is the X-ray spectrogram characteristic of the ferrous oxide. Further heating, without oxidation, causes the catalytic and magnetic properties to disappear simultaneously and the material becomes identical with haematite in all respects. We have here further evidence that we are to attribute the catalytic properties—and the magnetic properties—not so much to a definite state of oxidation of the iron as to a peculiarity of molecular structure which is inadequately conveyed by the valency exerted by the iron atom. The nature of this is not clear although we may suspect that it is akin to the co-ordinated structures which Baudisch has given to ferrous hydroxide, Manchot to the soluble catalytically active compounds of the type of sodium pentacyanoaquoferroate and Conant to haemoglobin and, by inference, to cytochrome—the suspected universal respiratory catalyst of Keilin.

Many studies have been made of the poisoning of enzymes by salts of the heavy metals. The latter are active in low concentration and their effects are frequently reversible. Some of these observations have led to interesting conclusions. Thus Euler[4] has found that salts of mercury and of silver inactivated his preparations of invertase and that the inactivation was complete when amounts of the heavy metals added were molecular equivalents of the amount of organic phosphoric acid in the enzyme preparation. It is a rea-

[17] Baudisch, O., *J. Biol. Chem.*, **61**, 261 (1924); *J. Am. Chem. Soc.*, **45**, 2972 (1923).
[18] Conant, J. B., *J. Biol. Chem.*, **57**, 401 (1923); **62**, 595 (1925).
[19] Keilin, *Proc. Roy. Soc. (Lond.)*, **98B**, 312 (1925).

sonable conclusion that there is a close relation between the activity of invertase and the organic phosphoric acid grouping in the enzyme.

Willstätter, however, does not agree that phosphorus is an essential constituent of this enzyme.

One other case of an attempt to identify the active group in an enzyme merits reference. K. G. Falk[2] is persuaded that lipases are protein in character and suggests that their activity resides in the **enolised form of the** peptide linkage which must, therefore, be present. It is demonstrated that lipase is inactivated by conditions which prejudice the enolisation of this group and that proteins and peptides can exhibit some lipolytic activity under conditions favoring enolisation. The experimental evidence is not convincing but the suggestion is intriguing as it presents a unique picture of an enzyme in the sense of Willstätter, in which the active catalytic group and the carrier are both intrinsic to the one molecule. It presents the enzyme as a protein phase on whose surface exists discrete points of catalytic activity—the peptide groups.

THE SPECIFICITY OF ENZYME ACTION

There has been the temptation amongst biologists to magnify the specific nature of enzyme activity. It has even been suggested that every biological reaction has its own special enzyme. The extravagance has been harmful to the co-ordination of data. If a preparation exhibits two distinct catalytic activities, are we to attribute these to two enzymes or to two potentialities of a single system? Unless we are able to separate the two activities from one another, we have only the measure of our experience to make the decision. It is not sufficient, as is often suggested, to destroy one effect and leave the other intact, for such treatment fails to discriminate between the specific imposition of unfavorable conditions and the specific activities themselves.

A reasonable conclusion which our accumulated knowledge affords is that nature provides catalysts adapted each to a particular species of reaction. One will accelerate the hydrolysis of α glucosides, another of β glucosides, a third of fructosides and so on. One will catalyse the opening of the peptide linkage between certain amino-acids, a second will be required for other peptides, whilst, in other cases, none can be found which are effective. The extensive researches of Abderhalden on the hydrolysis of synthetic dipeptides have clearly emphasised this. The differences observed submit to no classification at present, and here will be noted only the extraordinary discrimination of enzymes in respect to chemical differences restricted to structural disposition of identical groups about an asymmetric carbon atom. We are driven to the conclusion that optical asymmetry is also the general property of the active catalytic areas of the enzyme surface and to the conjecture that, associated with this, there will be a high degree of "asymmetric" orientation of optically active adsorbed molecules. That this is no fanciful exaggeration must surely follow from the fact that in the crystallisation of a racemic mixture the two enantiomorphs frequently show complete discrimination; the molecules of each isomer being exclusively adsorbed to and built up into the crystals of their own kind.

What seems to be required is the proof that an asymmetric surface can selectively adsorb one of two optically active isomers. Porter and Ihrigg[20] have reported such a case. They find that wool may be selectively dyed by

[20] Porter and Ihrigg, *J. Am. Chem. Soc.*, **45**, 1990 (1923).

the isomeric forms of certain asymmetric dyes which they have prepared. This observation has been questioned although the authors claim to have confirmed it.   The problem is of sufficient importance to deserve the most careful confirmation and extension.

Perhaps the most valuable contribution of Langmuir's theory of adsorption is that of the concept of the spatial orientation of adsorbed molecules. We are taught to appreciate that the adsorbed molecules are definitely oriented in space.   That is to say the surface complex has a definite chemical space structure.   Novel as this view may appear it is legitimate to regard it merely as an orderly extension of classical ideas of structural chemistry.   Therein we may expect to find the counterpart of steric hindrance, optical asymmetry and the like.   If, then, these surface complexes may be regarded as possessing definite chemical structure, we shall expect them to exhibit individual peculiarities in chemical reactivity; and the decision whether a surface will catalyse the decomposition of a substance which it adsorbs will be decided by the reactivity of the adsorption compound formed with the surface groups.   This may not immediately solve the problem of enzyme specificity but it possesses the merit of placing the onus for the specificity as much on the peculiar chemical properties of the reacting molecules as on the specific nature of the catalytic surface.   An attractive feature of the theory of Quastel, to which reference has already been made, is that it emphasises this aspect of specificity.

Some recent work of R. Kuhn and G. E. Grundherr [21] gives real substance to these conjectures.   The fact has already been quoted [10] that, of the products of hydrolysis of sucrose, laevulose is a much more potent inhibitant of the activity of yeast invertase than is dextrose.   Kuhn supplements this fact by the observation that in the case of the invertase derived from *Aspergillus,* it is dextrose and not laevulose which is the specific inhibitant of the enzyme.   Clearly these cases do not represent a simple displacement of the reactant from the surface of the catalyst by the products of the reaction catalysed.   Rather do they indicate a chemically specific poisoning of the active areas by *one* of the products of the reaction.   The simplest explanation would appear to be that the catalytic activities of the two enzymes are due to the formation of a complex with the sucrose.   In the case of yeast invertase this complex is formed with the laevulose portion of the sucrose molecule, and in the case of *Aspergillus* invertase with the dextrose portion. Kuhn offers an ingenious confirmation of this surmise.   He observes that while yeast invertase will effect the hydrolysis of the trisaccharide raffinose it is without action upon melezitose.   *Aspergillus* invertase, on the other hand, confines its activity to the latter sugar.   Now the structures of these two trisaccharides may be described by the abbreviated formulas, galactose-dextrose-laevulose (raffinose) and dextrose-laevulose-dextrose (melezitose).

A structural explanation for the specificity of the two enzymes immediately suggests itself.   In brief, this explanation is that the enzyme is active only if its point of union with the reacting molecule is readily accessible. In the two unfavorable cases which have been quoted we see that this is not the case.   An example of the same structural hindrance to enzyme activity is suggested by the old observation of Armstrong that galactose is a specific inhibitant of the activity of the lactase derived from bitter almonds, whereas the same enzyme obtained from yeast is inhibited by dextrose but not by galactose.

[21] *Ber.,* **59** [B], 1655 (1926).

In these extremely interesting observations the familiar "lock and key" simile which E. Fischer employed to describe the specificity of enzymes, materializes in definite chemical structures.

In this brief article the writer has been content to omit entirely many important phases of enzyme action. A confidence has been assumed which will scarcely survive a plunge into the literature of enzyme action. Many difficulties have been ignored and a strict consistency of treatment has been sacrificed to an attempt to present a simple picture of the complex physico-chemical system which we label "an enzyme."

The Adsorption of Enzymes

By Prof. Dr. Richard Willstätter,[*]

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Many enzymatic processes occur in ordered coöperation in unicellular organisms, and in every individual cell of more complicated organisms during their life-process. The agents responsible for enzymatic activity may be either known organic substances whose specific properties depend on their degree of dispersion, or the enzymes may be specific substances whose structure provides a future problem for organic chemistry. The second possibility seems more plausible; the quantity of enzyme as measured by enzymatic reactions, e.g., by use of a fungus or an organ, may be followed quantitatively to preparations having a thousandfold activity, in many cases without destruction or loss, despite the very great changes in degree of dispersion to which the enzyme has been subjected in the course of purification. Ordinary chemical methods cannot be used to isolate these high-molecular substances, which occur in as minute a percentage as radium in pitchblende; for with the exception of their specific reactions on their own particular substrates, the enzymes show no distinctive chemical characteristics, no clearly defined basic or acidic properties, and no chemical group reactions. So far, definite chemical reactions, e.g., precipitation reactions with mercuric chloride, phosphotungstic acid, etc., which are ascribed to the enzymes, fail to occur with increasing purification (as has been demonstrated in most instances), and after the separation of certain accompanying substances, they cease to be characteristic for the enzymes. A method of increasing the purity of enzymes which is fairly efficient, and up to date the only one generally applicable, is the adsorption of the enzyme by means of surface-active substances—a method which has been demonstrated and developed in recent researches on peroxydase,[1] saccharase,[2] pancreas lipase,[3] and similar substances. Some of the adsorbents suitable for this method are inorganic substances: aluminium hydroxide, ferric hydroxide, stannic hydroxide, kaolin, silicic acid, calcium phosphate, etc., whereas others are organic substances insoluble in water, such as cholesterin and tristearin. This method yields similar results in the isolation of other physiologically active substances, such as the products of internal secretions, toxins, antitoxins, etc.

It will indeed be important as regards the various applications of the method, to consider the limits which it must inherently reach. The adsorptivity of aluminium hydroxide, kaolin, etc., as opposed to the high-molecular organic compounds, is not selective enough, and therefore not satisfactory for sepa-

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[*] Translated by Eleanor Gertrude Alexander, M.A.
[1] R. Willstätter, *Ann.*, 422, 47 (1920-21); R. Willstätter and A. Pollinger, *Ann.*, 430, 269 (1922-23).
[2] R. Willstätter and F. Racke, *Ann.*, 425, 1 (1920-21), and 427, 111 (1921); R. Willstätter, J. Graser and R. Kuhn, *Z. physiol. Chem.*, 123, 1 (1922); R. Willstätter and W. Wassermann, *Z. physiol. Chem.*, 123, 181 (1922-23); R. Willstätter and K. Schneider, *Z. physiol. Chem.*, 133, 193 (1923-24), and 142, 257 (1924-25).
[3] R. Willstätter and E. Waldschmidt-Leitz, *Z. physiol. Chem.*, 125, 132 (1922-23).

rating mixtures. Since enzymes are also found in the form of rather complicated mixtures of substances, we must not expect that our knowledge of adsorption phenomena will be materially increased, but only that the sphere of their application may be appreciably extended.

The method of separating enzymes by means of adsorption has a long history, but the earlier experiments seem to have been so unsatisfactory that a few years ago O. Hammarsten and S. G. Hedin [4] concluded: "The adsorption process is more or less irreversible and thus differs from the adsorption of crystalloid substances." One of the first attempts in this direction was described by J. Vogel (1844),[5] who precipitated a pepsin solution with lead acetate, and from the albuminous precipitate dissolved out the adsorbed pepsin by treatment with H_2S and water. Another one of the earliest attempts was made by E. Brücke (1861) [6] also with pepsin, which he "adsorbed mechanically on small solid particles," i.e., calcium phosphate, sulfur, or cholesterin. Immediately thereafter, A. Danilewsky [7] in Kühne's laboratory tried to separate out the three physiologically active substances of pancreatic juice by adsorption methods, and J. Cohnheim [8] made a similar attempt with salivary amylase and pancreatice amylase.

Only forty-five years later did the adsorption method gain a new impulse through the researches of L. Michaelis [9] and L. Michaelis and M. Ehrenreich,[10] "The Adsorption Analysis of the Ferments." With the intention of observing pure electrochemical adsorbents, Michaelis and Ehrenreich chose adsorbents which "under all conditions were oppositely charged." The adsorption phenomena were then referred to as the opposed electric charges of adsorbate and adsorbent. Thus it was established "that all substances adsorbable by kaolin must be bases; all substances adsorbable by alumina must be acids." According to this guiding principle, the electrochemical nature of the ferments could readily be established as the basis of evaluating their reactions.

"Invertin is adsorbed by alumina, whatever the reaction, but not by kaolin in any reaction; it thus has all the properties of an acid."

"Salivary diastase is adsorbed under all conditions by both kaolin and alumina, and is thus an amphoteric substance."

"Trypsin is adsorbed by kaolin and alumina (i.e., completely) when the reaction is neutral or acid, and is, therefore, an amphoteric substance."

Nevertheless, contrary to these views and statements, it has been shown that these facts do not apply to the enzymes themselves; that they do not indicate anything at all with regard to their nature, but apply only to crude enzyme solutions with varying aggregates of enzymes and accompanying foreign substances. And even for the impure solutions, these statements are not confirmed. R. Willstätter and F. Racke [11] showed that invertin, after certain purifying processes, is easily adsorbed by kaolin and further researches have shown that under certain conditions, it is immediately and quantitatively adsorbed from yeast autolysate by kaolin. This enzyme, in the degree of purity thus far obtainable, acts like an amphoteric substance.

A decided step in the improvement of the adsorption method, was finding

[4] O. Hammarsten, "Lehrbuch der physiologischen Chemie," 1922, 9th ed., p. 38.
[5] J. Vogel, *Jahresber. Berzelius*, 23, 606 (1844).
[6] E. Brucke, *Sitzb. Akad. Wiss., Wien*, 43, 601 (1861).
[7] A. Danilewsky, *Virchows Archiv*, 25, 279 (1862).
[8] J. Cohnheim, *Virchows Archiv*, 28, 241 (1863).
[9] L. Michaelis, *Biochem. Z.*, 7, 488 (1907-8), and 12, 26 (1908).
[10] L. Michaelis and M. Ehrenreich, *Biochem. Z.*, 10, 283 (1908).
[11] R. Willstatter and F. Racke, *Ann.*, 425, 1 (1920-21), and especially 55.

a way to separate the enzyme from its adsorbent. The adsorption of the enzyme, i.e., on alumina or kaolin, is not, as a rule, a reversible process like the adsorption of acetic acid or acetone by animal charcoal. When, for example, invertin is adsorbed by aluminium hydroxide out of a slightly acid solution, after separation from the mother-liquor, the adsorbate yields practically no enzyme substance to dilute acid of the same acidity. Shortness of reaction time may be a factor in the process—a fact which is taken advantage of in washing off the adsorbate. In many cases a mere change of reaction in the medium is sufficient to loosen up the adsorbate. An important modification of this method is the use of different solvents for the adsorption and release of the enzyme, in order to influence the distribution of the enzyme between solvent and adsorbent, and thus favor adsorption in the desired direction. Adsorption is favored in alcoholic solution, elution in aqueous solution. The observed difference in behavior in aqueous and alcoholic or acetone solutions, depends on the fact that organic compounds such as the amino-acids and petids lose their basic properties in alcoholic solution, so that their basic salts react as neutral.[12] The dominance of acidity is very favorable for adsorption on basic aluminium hydroxide. In this instance there are two exceptions. In the case of peroxydase,[13] the adsorption in alcoholic solution seems to be accounted for by the properties and constitution of the enzyme itself, which in all known degrees of purity is hardly adsorbed at all by alumina in aqueous solution, thus behaving itself like an amino-acid. Another case observed is that of pancreas amylase.[14] Here behavior depends on the mixture of peptid-like accompanying substances. With increase in enzymatic purity, the amylase loses its property of being readily adsorbed in alcoholic solution, and even loses entirely the property of adsorbability by aluminium hydroxide or kaolin. In order to characterize an enzyme with respect to its adsorbability, it must be tested comparatively with the individual adsorbents in various and highest attainable degrees of purity.

Besides change of solvents for adsorption and elution, there is only one general method of isolating enzymes chemically. As adsorption behavior depends upon small specific attractions and residual forces, the adsorbate can be loosened more readily if these are overcome. Very dilute alkali is sometimes suitable for this—as for example weak ammonium hydroxide or very dilute sodium hydroxide, or better yet, dilute solutions of disodium phosphate. When ammonia or soda is used to liberate invertin from alumina, the action is materially aided by the additional presence of the phosphate ion. When tertiary calcium phosphate has served as adsorbent, the enzyme is liberated by changing the precipitate to a secondary phosphate. Various enzymes in a fairly purified state, and at times even in the crude state as obtained in organ-extracts or autolysates, show a definite gradation in their adsorptive behavior which makes it possible to separate one from another, as for example, the different components of pancreatic enzyme mixture. Pancreas lipase [15] is very easily adsorbed, and is thus not held so closely by accompanying substances. It is adsorbed as easily by electro-positive as by electro-negative adsorbents, by alumina and kaolin, and is readily released by alkaline phosphate solution—best of all if it contains glycerin. Amylase and trypsin stand out in contrast

[12] R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, 54, 2988 (1921); F. W. Foreman, *Biochem. J.*, 14, 451 (1920).

[13] R. Willstätter and A. Pollinger, *Ann.*, 430, 269 (1922-23), and especially 273.

[14] R. Willstätter, E. Waldschmidt-Leitz and A. R F Hesse, *Z. physiol. Chem.*, 126, 143 (1922-23), and 142, 14 (1924-25).

[15] R. Willstätter and E. Waldschmidt-Leitz, *Z. physiol. Chem.*, 125, 132 (1922-23).

because of their stronger acid properties. Therefore lipase can be separated out from the two accompanying enzymes, and even quantitatively, with practice. As amylase lacks basic properties, it, furthermore, can be separated from trypsin, which is readily adsorbed by kaolin. In order to increase the purity of an enzyme by use of the adsorption method, the adsorption must be carried on as quantitatively as possible. In general, the less adsorbent required to take up a given enzyme, the purer the enzyme held in the adsorbate. It is, therefore, essential to choose a suitable preparation of adsorbent. The adsorptive behavior of alumina, for example, depends upon the sort used. According to the manner in which they precipitate out from aluminium salts, hydrates [16] of aluminium oxide have been distinguished, which differ in physical state and adsorptivity, and which are called A, B, and C. Aluminium hydroxide C has the formula $Al(OH)_3$; B and A are more dehydrated forms. The standard used in the selectivity of the adsorption is the adsorption value A.V.[17] It signifies the quantitative unit of an enzyme, which under stated conditions is adsorbed by 1 gm. of adsorbent. With gels such as metal hydroxides, this information is useful with regard to water free oxides, i.e., Al_2O_3. In the researches with invertin (saccharase), where the method has been best worked out and understood, we are at once struck by the extraordinarily great influence exerted by the purity and previous history of the enzyme solutions used, namely autolysates of yeast. When the method for isolating invertin from yeast had been improved, and the most suitable sorts of aluminium hydroxide had been found, the adsorption values reached for aluminium hydroxide were 0.15-0.2. Further attempts resulted in discovering that alumina, upon greater dilution, adsorbs invertin from impure solutions much more selectively, and that its adsorption value for invertin becomes very much greater.

Invertin Units	Al_2O_3	Amount Water Containing 1 Unit of Invertin	Adsorption Per Cent	A.V.
0.121	0.186	400 ccm.	9	0.059
0.121	0.1395	400 "	19	0.16
0.121	0.1395	20000 "	93	0.81

Therefore we find, in agreement with past experience, that a certain quantity of adsorbent will take up relatively more substance from dilute solutions than from more concentrated ones; further, the absolute quantity of adsorbed enzyme rises upon diluting the solution, and the adsorption thereupon becomes more highly selective for invertin than for the major portion of the accompanying decomposition products of the protoplasm. Moreover, in each case, the zone of optimum acidity for adsorption must be found. Through observing the adsorption process quantitatively, it has become possible, with increasing purity of invertin, to raise the adsorption value of alumina for this enzyme to more than a thousand times the first observed value, so that invertin is taken up with an A.V. of alumina equal to 200. After the first improvement attained, invertin was taken up from 10 grams of living yeast by 1 gram of

[16] R. Willstätter and H. Kraut, *Ber.*, **56**, 149 (1923), and **56**, 1117 (1923), and **57**, 58 (1924), and **57**, 1082 (1924).
[17] R. Willstätter and F. Racke, *Ann.*, **425**, 66 (1920-21), and R. Willstätter and W. Wassermann, *Z. physiol. Chem.*, **123**, 184 (1922).

Al_2O_3 and after the last improvement from 12,000 or 14,000 grams of yeast.[18] In this case the invertin adsorbate formed from 1 gram Al_2O_3 weighed 2½ grams, and the alumina adsorbate has an invertin content 4500 fold more concentrated than the fresh yeast used. Thus by adsorption invertin can be obtained 2,000 times more concentrated than in dried yeast; peroxydase 12,000 times more concentrated than in dried plant root; and lipase 300 times more concentrated than in the desiccated and de-fatted gland.

The adsorption behavior of the enzyme in impure solution is dependent on (1) foreign material mixed with it and (2) the accompanying substances closely bound up with it which may be described as coadsorbents. Systematic fractional adsorption separates an enzyme thoroughly from group (1), but not from the substances of group (2). Therefore it is necessary to investigate the laws governing enzyme adsorption from impure solutions. This investigation was accomplished in the noteworthy researches of H. Kraut and E. Wenzel,[19] whose work should be looked up as it can only be briefly mentioned here. In graph form, the adsorption value is used as ordinates, the concentration in units per liter in the remaining solution as abscissas. While the adsorption isotherm of a simple substance in dilute solution is represented by a parabola, the form of the curves in the crude enzyme solutions are found to be diverse and anomalous, because the adsorption takes place from a mixture of substances. In this case, all the adsorbable substances present, distribute themselves on the surface of the adsorbent in accordance with their degree of adsorbability. The adsorbability of each substance depends upon its concentration and its affinity for the adsorbent. Changing the original concentration of an enzyme solution results in a relative change in the concentration of all substances in solution; but like changes in the *concentration* of the various substaces bring about totally unlike changes in their *adsorbability,* so that each substance is displaced from its maximum of adsorbability, and is thus brought into a superior or an inferior position relative to the adsorbability of the enzyme. The form of the adsorption curve shows that fractional adsorption follows the removal of the accompanying substance (in a favorable or an unfavorable sense). Thus, by partial adsorption, most of the accompanying substances are removed, with a small amount of the enzyme; or a large portion of the foreign material is left behind in the mother liquor, with a residual quantity of enzyme. Moreover, in crude enzyme solutions the hydrolytic splitting off of loose accompanying products composed of enzymes and foreign material, is favored by greater dilution and acidification; and with enzymes of higher purity, the breaking up of systems composed of enzymes and co-adsorbents closely bound to the adsorbate, is also effected by dilution and the addition of acid.

The object of this application of the adsorption method is to purify the substance to such an extent that its adsorption isotherm is that of a pure substance. With invertin solution, this is attained by repeated adsorption on kaolin and alumina. There is some question as to the degree of purity of the enzyme thus obtained, that is, whether it is a definite chemical substance. Even when the behavior of invertin on adsorption makes it seem homogeneous, it is still apparently far from being a pure substance. The preparation and analysis of invertin specimens in this fashion are important as regards certain decomposition products of albumin and peptides, which can be removed under

[18] R. Willstätter and K. Schneider, *Z. physiol. Chem.*, 142, 262 (1925).
[19] H. Kraut and E. Wenzel, *Z. physiol. Chem.*, 133, 1 (1923-24), and 142, 71 (1925-26).

certain conditions, and therefore are not an integral part of the chemical structure of invertin.   H. von Euler and K. Josephson,[20] on the basis of the analysis of their invertase preparation, expressed the view that the peptide tryptophane has an important place in the structure of invertin; but R. Willstätter and K. Schneider [21] showed that though purified invertin may carry 9 per cent of tryptophane to the end of the process described above, nevertheless, under certain conditions (e.g., by fractional precipitation with lead acetate) it is possible to free invertin substantially from tryptophane.

Thus the adsorption curve of a purified invertin containing tryptophane, is not that of invertin itself, but is the curve of an impure invertin, or, more specifically, that of an aggregate consisting of invertin and its most closely related substances (such as other enzymes and the decomposition products of enzymes) adsorptively bound with one or several co-adsorbents.   What has been accomplished is not the desideratum of isolation of an enzyme, but rather a foundation for deeper, more penetrating investigation.   By changing conditions of adsorption, e.g., the concentration and acidity of the solution, the attempt must be made to influence the association of the enzyme with its co-adsorbent in such a manner that either part of the accompanying substances or else the invertin itself will be selectively adsorbed by the same adsorbent, or more readily still by other adsorbents.

In all applications of the adsorption method, this principle should be considered also for the purification of other substances of unknown constitution. Various high-molecular compounds may occur more or less closely bound to such aggregates, and continue to be bound during the adsorption process, so that there is a semblance of unity.   Through comparative investigations with various samples of the same substance, and through different adsorption conditions, it will be possible to examine closely the individuality of high-molecular organic compounds, and to purify them still further.

[20] H. von Euler and K. Josephson, *Ber.*, **56**, 446 and 1097 (1923); **57**, 299 and 859 (1924); *Z. physiol. Chem.*, **138**, 11 and 38 (1924).
[21] R. Willstätter and K. Schneider, *Z physiol. Chem*, **142**, 257 (1924-25).

The Present Status Attained by Colloid-Chemical Investigation of Peptide-Splitting Enzymes *

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I. Introduction

On Stoichiometric and Non-stoichiometric Compounds

Our knowledge of the nature of enzyme action is on the eve of great advances. We are at a new line of departure in the technique of enzyme investigation, and new methods lead to new knowledge. In the older methods of enzyme research, physico-chemical investigations were carried out either on living cells, or on press juices or cell extracts; but there were no systematic methods available whereby these extracted substances could be purified. With the development of purification methods, new vistas opened up.

We shall first review the developments of earlier enzyme research, omitting antiquated views such as prevailed about the time of Liebig and Pasteur, and considering only relatively modern research beginning about the time of Buchner's discovery of cell-free fermentation, in 1898. His discovery that sterile yeast press juice would ferment sugar into alcohol and CO_2 (though under quite different concentration than the living yeast), led to the gradual transfer of the problem of enzymes from the biologist to the chemist. It became necessary to discover the active constituents of the press juice, and to investigate their specificity with regard to the substrates they split. Thus arose two different research objectives: the chemical nature of the enzymes, and their mode of action.

The first of these problems has been quite neglected for lack of suitable methods, while work on the second was extensively carried out by Emil Fischer and his school, principally with enzymes which hydrolyze carbohydrates and proteins, and their derivatives. The results of these investigations relative to the specific character of enzyme action, are well known, and are summed up in Fischer's famous comparison to lock and key.

Besides these specificity experiments, physical chemists made kinetic measurements to see if enzyme action could be referred to a catalysis like that, e.g., of H-ions in the inversion of cane sugar, where according to Wilhelmy, the velocity of inversion follows the theoretical laws of catalysis, and is strictly in proportion to the H-ion concentration, the splitting of the sugar itself being a monomolecular reaction.

But with enzymes such an agreement with theories of catalysis and monomolecular splitting was only exceptionally found, and even then only under very restricted conditions of enzyme and substrate concentration. Sørensen tried to account for this general deviation from the theory, by introducing

* Translated by Dr. Marta Sandberg.

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a constant reaction of the milieu with the help of regulators (buffers), but with little success. The main result, however, which emerged by keeping constant the reaction of the enzyme milieu, was not that the laws of catalysis were ideally exemplified, as had been generally expected. On the contrary, it was shown that variation of reaction occasioned a simultaneous change in the kinetic course of the reaction.

Now this fact found an analogy in experiments which Bredig and his co-workers had carried out in 1899 on catalytic decomposition of hydrogen peroxide by colloidal platinum. Here also the kinetic curve (that is, time plotted against decomposition), were dependent on the reaction of the medium. It was suggestive to conclude from these experiments in a definite colloid system, that in enzyme systems too, there function certain colloids which are susceptible to change of reaction. It must here be recalled that nearly all colloidal systems react strongly to any addition of electrolytes. Depending on whether the colloid in question is of a basic or of an acid nature, it will show a high susceptibility to OH-ions, or H-ions. Even a very small excess of OH-ions coagulates a solution of colloidal iron oxide. Solutions of globulin or of phospho-protein (of yeast, pancreatic juice, casein, etc.), colloidal solutions of arsenic trisulfide, and generally of all colloids and suspensions which would naturally migrate to the anode during cataphoresis, are highly susceptible to small amounts of H-ions, and are coagulated by them. As to other anions and cations, it is well known that their action on colloids may differ greatly. Some powerfully coagulate certain colloids, but act less strongly on others. Suspensions of clay, for instance, are strongly coagulated by small amounts of neutral salts; globulins, on the other hand, are dispersed by the same salt under the same conditions of concentration. Neutral phosphates disperse suspensions of clay as well as proteins; obviously by a specific action of the phosphate ion.

According to H. Freundlich,[1] the electric charges of the colloidal particles are mainly responsible for these actions of electrolytes. Those ions of electrolytes which carry opposite charges, discharge and therefore coagulate the colloid. But at the same time the specific adsorption of active ions at the surface of the colloid, and their valence, play a decisive part. Those ions which are most strongly adsorbed and which carry the higher charges, are most potent as coagulants.

But according to the author's view, electric phenomena cannot occupy the commanding place that corresponds to the now generally accepted theory of Freundlich. Primarily there are two conditions to be considered: first, whether the action of the coagulating electrolyte is due solely to the fact that it penetrates into the hydration layer of the colloid particles and is thus adsorbed (lyosorption); or whether the chemical substance of the colloid enters into chemical combination with the electrolyte or with its ions (chemosorption). Both cases, between which transition phenomena occur, must be considered separately. If a suspension of clay is coagulated by the action of neutral salts, we may have to deal only with lyosorption. The salt penetrates into the hydration layer, which surrounds the particles of clay, gradually breaks up its adhesion to the substance of the particles, and thus produces desolvation and aggregation of particles. On the other hand, neutral phosphates, which are just as well adsorbed and therefore also penetrate into the hydrate covering, not only do not coagulate clay suspensions, but even stabilize them.

[1] Freundlich, H., "Kapillarchemie."

But these facts are not inconsistent with specific action of ions which produce coagulation, or stabilization. Using alkalis which, according to the discharge theory of coagulation, act by specific OH-ion influence, we get quite a different picture. If we add ammonia and sodium hydroxide to clay suspensions, absolutely different effects are found though, according to the theory of ion action with subsequent discharge, in both cases the OH-ion might be the active constituent. Ammonia produces suspension independent of concentration, but sodium hydroxide, when added in increasing amounts, produces coagulation. It is, therefore, not a question of the action of the OH-ions alone; the whole electrolyte participates in the effect. In the case of lyosorption, according as the electrolyte decreases the strength of adhesion of the hydration-covering or not, we have coagulation or stabilization. The different action of the two substances that show so much similarity can hardly be imagined in any other way. The dissociation coefficient of the two reagents cannot be significant, for the discharged OH-ions unbalance the dissociation equilibrium when ammonia is used and fresh OH-ions are produced. Electrolytes penetrate into the hydration layer, decrease, and even abolish completely its strength of adhesion, while other electrolytes, though penetrating into this layer, leave the covering intact and add to the stability of the colloidal system.[2]

Proof of the correctness of suggesting the existence of lyosorption with subsequent coagulation may be found in the fact that the same electrolytes which even in low concentration coagulate slightly solvated (lyophobe) sols, must be in considerable concentration in order to coagulate strongly solvated (lyophile) sols. Gold sols are coagulated by very small amounts of salt, strongly solvated globulin sols, however, require semi-saturation with ammonium sulphate, and albumin sols (which are still more strongly solvated) require full saturation.

With the second type of concentration of substances at the surface of dispersed particles, their solubility in the solvation layer of the dispersoid is only of secondary importance and the question of dehydration need not be considered at all.

The chemical affinity of the surface of the colloidal particles, is here the decisive factor, and it is supposed to be usually identical with the chemical action generally exhibited by acids and bases, or between these substances and salts. Silicic acid or stannic acid gel is dispersed or peptized, as this process of dispersion has also been called by Thomas Graham, by very small amounts of alkali. Here we see evidence of the chemical affinity between an acid and a base. But there exists a difference which, if not qualitative, certainly is quantitative between the cases where molecularly dissolved acids and bases react, and where a molecularly dissolved base is combined with a colloidally dispersed acid. The first instance is controlled by Dalton's law and by stoichiometry, which is based on it. But the well known laws of constant and multiple proportions fail when chemical valences, instead of being those of true chemical molecules, of a dimension of about 10^{-8} cm. act on colloidal particles, which aside from their active surfaces, have a variable inactive mass. From a topochemical point of view, only those groups of atoms in colloid molecules will react, which are in the surface accessible to the reagent. The laws of chemical quantities and masses, upon which molecular chemistry is based, are not applicable to colloidal molecules, wherein we

[2] Fodor, A., "Die Grundlagen der Dispersoidchemie" (Dresden, 1925).

must distinguish between surfaces capable of reaction and the inactive inner mass.

If, therefore, a "gel," i.e., a colloid precipitate non-dispersible in water without some addition, is dispersed by combining the active surface with a dispersator or peptizer of true molecular nature (chemosorption means just this combination), the intensity of combination depends on how large the active surface of the colloid precipitate is at the outset. Generally substances react quicker, the smaller the size of their granules. A finely flocculent precipitate, therefore, will react more readily with a dispersor than a coarse or dried mass of the same colloid. The same colloid mass, therefore, differs as to its chemical quantitative value according to its amount of free, active surface. The dispersor produces new surfaces at dispersion, but this need not always happen. From study of the colloid chemistry of proteins, we find we must distinguish between two actions: (a) the power of the colloid to combine with the dispersor and, (b) the power of dispersion possessed by the same dispersor. The first of these two factors effects chemosorptive combination, the second effects the solubility of the colloid. Both may be observed separately and independent of each other.

There exist, for instance, proteins that are insoluble in water, but swell strongly when treated with acids or bases, which shows that the protein combines with the electrolyte. The solution, i.e., the dispersion of the protein, however, does not occur at concentrations which cause a swelling. Here we are dealing with quite different conditions than those which control the solution of a difficultly soluble crystalline acid by a base, where solution occurs in proportion to the reaction of the base with the acid, while the mixture is stirred in order to facilitate diffusion. In this case the salt that has been formed is dissolved. If the same thing would happen with colloidal precipitates, a certain amount of alkali, for instance, should combine with the equivalent amount of stannic acid and dissolve it by forming a stannate. The large amount of colloid, however, would remain unchanged. That this possibility is not realized is shown by the fact that even minimal quantities of alkali disperse *all* the stannic acid and hold it in colloidal solution.

This phenomenon can be explained only by adsorption of the added alkali or of the stannate formed at the *whole* surface of the peptized stannic acid. Which of those two possibilities is actually the case in each particular colloidal system, cannot always be ascertained. Some authors, as R. Zsigmondy [3] and Wo. Pauli, [4] believe that the surface is always adsorptively charged by salt-like compounds of the colloid substance, like the stannate in the above mentioned instance, or salts of metal oxides in iron hydroxide sol or in similar colloids. The writer believes that in both cases it is a matter of hypothesis if we attribute to the stannic acid sol the symbol

$$
\text{stannic acid} \quad
\begin{array}{ll}
-\text{OH}^- & \text{Na}^+ \\
-\text{OH}^- & \text{Na}^+ \\
-\text{OH}^- & \text{Na}^+ \\
\vdots & \vdots
\end{array}
$$

or

$$
\begin{array}{l}
\text{stannic acid} - \text{stannate ion}^{----} \text{Na}_4{}^{+++\,+} \\
\qquad\qquad\quad\; - \text{stannate ion}^{----} \text{Na}_4{}^{+\,+\,+\,+} \\
\qquad\qquad\qquad\qquad\quad \vdots
\end{array}
$$
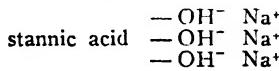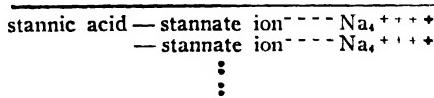
[3] Zsigmondy, R., "Kolloidchemie."
[4] Pauli, Wo., see his paper in this volume.

With proteins, however, we are justified in accepting the former possibility. For if we peptize a solid protein by acid or alkali, or add one of these substances to a protein solution, an acid- or alkali-protein is formed which shows all the properties of colloid complexes and which must possess the same structure as the stannic acid complex just described. (These colloid complexes which are also called micells, show amongst other properties, a remarkably high electric mobility which is connected with their peculiar structure, especially with the accumulation of foreign ions at their surfaces.) If the solution of an acid- or alkali-protein is mixed with the solution of a neutral salt, we observe the release of H- or OH-ions by the protein. Accordingly the acid must have been present in the acid protein complex in a latent form and the same refers to the alkali in the alkali-protein complex. If H-ions disappeared on combination of acid with protein, as with neutralization of a base by an acid, then we would expect no splitting off of acid by the addition of salts. But if the acid protein compound is supposed to contain the acid in a latent and therefore easily displaceable state because of hydrolytic splitting, it is, we think, entirely immaterial whether the adsorptive adhesion of the acid to the surface of the protein in a free state is explained by hydrolysis or not. The important fact is that there have disappeared from the solution H-ions which formerly could be estimated potentiometrically, but which are fixed at the surface of the protein in a free state. Besides, in these colloid systems hydrolysis is of quite subordinate importance.

Returning to our subject, we repeat that the peptizing substance must occupy the whole surface of the colloid, because the phenomenon of peptization cannot be explained in any other manner. If the peptizer would combine with an equivalent part of the colloid, perhaps according to stoichiometrical proportions, no peptization would result, but only a mixture of unchanged colloid *and* the combination. It is, therefore, necessary that either the peptizer itself or this compound (which acts as a peptizer) be distributed over the whole surface of the colloid.

But it is easy to understand that it cannot be a matter of indifference as regards the state of a disperse system, whether the peptizer distributes itself over a large or a small surface. It makes no difference whether the peptizing agent is the substance originally added, or its combination with the colloid. The real peptizer, up to a certain degree of saturation, occupies the whole surface, whether this is extensive or not, or whether the whole mass of the colloid is large or small. With proteins it has been established that the original degree of dispersity of the same quantity of protein at the time of combination with the same equivalents of alkali, is of great importance for the characteristic properties of the resulting alkali-protein systems. For if we combine a certain amount of alkali, once with a highly dispersed protein and another time with a less highly dispersed one, we get in the first case a solution of alkali-protein of a higher viscosity and lower electric mobility.[5] This phenomenon cannot be explained here.* It is obviously connected with the distribution of foreign ions at colloidal interfaces. But as proteins are usual constituents of cells and tissues, and are also important in judging the actions of enzymes (either because enzymes occur together with proteins or because in some cases enzymes seem to be proteins or as protein-like substances), we shall now give a brief review of the colloid chemical properties of proteins.

* Fodor, A., *lib. cit.*, and *Kolloid-Z.*, 1925-6.
* See, however, J. Alexander on the viscosity of hydrophile sols, *J. Am Chem. Soc.*, 1920. J A.

both directions. The external appearance of the solution changes little if at all during this process. But if the solution is heated to about 60° C., albumin coagulates.* This coagulation optimum of protein, therefore, shows a double charge, simultaneously anodic and cathodic. This condition has, therefore, been termed the isoelectric state.

The other proteins above mentioned show a somewhat different behavior when acid is added: as soon as a certain acidity is attained, they coagulate directly, i.e., without being heated. What happens to the enhydrone micells when they are exposed to the action of this small amount of acid? Where coagulating proteins are concerned, which Wo. Ostwald calls *"isolabile,"* the explanation is easy. Obviously, the enhydrone is dehydrated, i.e., the particles lose their water membranes, aggregate, and, therefore, coagulate. The case of "isostable" albumin is less easily explained. This also is on the point of coagulating, as we have seen, but actual coagulation occurs only upon heating. In this case also the enhydrone has been attacked, but on the other hand another influence seems to act, which prevents spontaneous coagulation. We shall not be mistaken if we connect this fact with the double migration, and assume that the acid added participates in the process. This acid confers a cathodic charge on the protein through its H-ions, as may be seen from the following symbols:

$$\left[\left[\text{albumin}\begin{array}{c}-OH^-\\-OH^-\\-OH^-\\\vdots\end{array}\right|\begin{array}{c}H^+\\H^+\\H^+\\\vdots\end{array}\right]\begin{array}{c}\longleftrightarrow\\\longleftrightarrow\\\longleftrightarrow\\\vdots\end{array}\begin{array}{c}H_2O\dots\\H_2O\dots\\H_2O\dots\\\vdots\end{array}\xrightarrow{HCl}\right.$$

albumin-enhydrones
(strongly hydrated)

$$\left[\left[\text{albumin}\begin{array}{c}OH^-\\OH^-\\OH^-\\\vdots\\H^+\\H^+\\H^+\\\vdots\end{array}\right|\begin{array}{c}H^+\\H^+\\H^+\\\vdots\\OH^-\\OH^-\\OH^-\\\vdots\end{array}\right]\begin{array}{c}\\\\\vdots\\\longleftrightarrow\ H_2O\\\vdots\end{array}\right].$$

isoelectric albumin
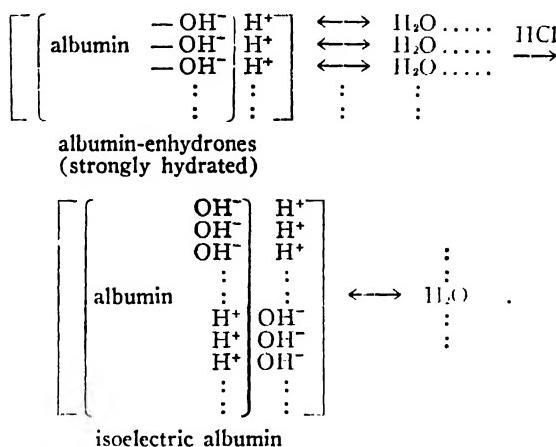
By choosing these symbols for the protein micells, we suggest that the whole free and active surface of the protein is considered as a unit, and not single particles, of which we at present know nothing.

We see that the original enhydronic charge of albumin has been increased by another charge by the H+-ions of the acid, the definite charge of the whole surface, therefore, being produced by OH--ions as well as by H+-ions. It is obvious that a very labile system will be the result; for these two kinds of ions discharge each other with the utmost facility. Nevertheless there exists a certain tolerance in this respect, caused, however, by unknown inner reasons. But only when the protein is heated does definite discharge occur, and coagulation takes place at the same time. With true globulins and phosphoproteins there exists obviously no possibility of a double charge of this kind,

* On the heat-coagulation of proteins, see paper by Mona Spiegel-Adolf, this volume. *J. A.*

which after all must be connected in some way with their small hydration capacity.

If an isoelectric solution of albumin is treated with further amounts of acid, or if the other flocculated proteins are thus treated, solutions with high internal friction will form, until the optimal addition of acid is reached, beyond which the viscosity decreases again. An entirely symmetrical process occurs, if we treat enhydronic proteins directly with alkali. Here also solutions of high viscosity are formed up to a maximum addition of alkali. But while acid protein migrates only to the cathode, alkali protein moves only to the anode. Neither of the two new protein systems, however, migrates when pure water is used as dispersion medium; therefore solutions of electrolytes have to be used in cataphoresis. The nature of these protein solutions of high viscosity is entirely different from that of enhydrones. What kind of micells do they contain?

In order to answer this question we must first investigate the most essential properties of these new protein systems. Their high viscosity implies a strong solvation. On the other hand when investigating the electrolytic properties of these systems, we find that they contain the protein in a state of high conductivity, which greatly exceeds the order of magnitude of ordinary organic ions. Only conductive carriers in soap-solutions, which have been studied by McBain,[7] * show a similar behavior in this respect. We, therefore, must be dealing with electrically charged protein-complexes of a high degree of hydration.

There remains only one question to be answered. We must decide whether these carriers of positive charges in acid proteins, or of negative charges in alkali proteins, correspond to ordinary organic ions with extraordinarily high electric migration, or if we are here dealing with special conditions.

Wo. Pauli takes the former view.* According to him polyvalent protein ions produce these high mobilities. But this is contradicted by a considerable number of experimental findings in this field, which do not agree with theoretical ionic views, valid for dilute solutions. The degree of solvation, for instance, which according to Pauli is in proportion to ionization, does not run at all parallel with the strength of the acid forming the acid protein. Lactic acid albumin is more strongly hydrated and, *ceteris paribus,* more ionized than sulfuric acid albumin, etc.

This shows that salt formation, such as occurs between acids and bases does not exclusively participate in the formation of acid and alkali protein. Additional evidence is offered by the following finding: experiments with yeast phosphoprotein, which is typically polydisperse, show that its ability to combine with alkali is not constant, even when the substances acting are used in exactly the same concentration. Combination depends rather on the state of dispersion of the protein. A coarser degree of dispersion of the protein produces, upon combination with the same amount of alkali, lower viscosity and higher mobility of the protein constituent of alkali protein, than when the protein is more finely dispersed. In this case the same combination with OH-ions causes stronger hydration and lower conductivity value.

We cannot, therefore, possibly be dealing with ordinary ion formation or with stoichiometrical relations of combination. Pauli's explanation, that

* See paper by J. W. McBain, Vol. I of this series. *J. A.*
[7] McBain, J. W., *Repts. British Assoc. Adv. Sci.,* also *J. Soc. Chem. Ind.,* **37,** 249 T. (1918), and his paper in Vol. I, of this series.
* Pauli's latest views are given in his paper in this volume. *J. A.*

is, his assumption of polyvalent protein ions, is not valid for the interpretation of the behavior of our protein systems; for although ions of soap solutions (palmitate, or stearate-ions, etc.) are certainly univalent, the electric carriers of soap solutions are distinguished by abnormally high mobilities. In both cases colloidal micells must be considered as responsible for these electric properties.

If we want to picture these micells, we must imagine that the surface of contact of the protein with the milieu is charged by H-ions or with alkali proteins by OH-ions, while in both cases the second ion remains in the free fluid as an "inert ion." * This interpretation corresponds to the theory of an electric double layer. These highly dispersed colloid complexes, which are charged by H-ions or by OH-ions, possess, therefore, high electric mobility and a powerful capacity for hydration. These properties are due to the specific nature of the ions, into which we will not enter here, especially as our knowledge in this respect is rather limited. In order to distinguish them from the above mentioned enhydrones they are termed as *ekhydrones,* because their electric charge is not produced by the ions of water, but by the ions of acids and alkalis. The water which has been attracted is therefore of secondary importance, and must be thought of as occupying, as it were, an exterior position.

The constitution of such a micell may be expressed by the following symbols:

$$\left[\left[\begin{matrix} \text{protein} & \begin{matrix} -\,OH^- \\ -\,OH^- \\ \vdots \end{matrix} \end{matrix}\middle| \begin{matrix} Na^+ \\ Na^+ \\ \vdots \end{matrix} \right] \begin{matrix} HOH\dots \\ HOH\dots \\ \vdots \end{matrix} \right.$$

or

$$\left[\left[\begin{matrix} \text{protein} & \begin{matrix} H^+ \\ H^+ \\ \vdots \end{matrix} \end{matrix}\middle| \begin{matrix} Cl^- \\ Cl^- \\ \vdots \end{matrix} \right] \begin{matrix} HOH\dots \\ HOH\dots \\ \vdots \end{matrix} \right.$$
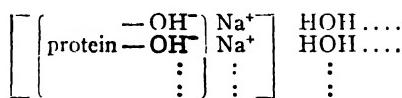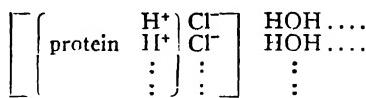
One of the most outstanding characteristics of protein ekhydrones is that they do not migrate in cataphoresis with pure water as the dispersion medium. But they begin to migrate in one direction as soon as the electrode space of the apparatus is filled with a suitable electrolyte solution.

One of the most important peculiarities of these ekhydrone systems is the fact that they cannot be adsorbed by animal charcoal, kaolin, etc. The same proteins in the enhydronic state, however, are easily adsorbed by these reagents. Likewise acid and alkali proteins cannot be coagulated by heating. Addition of electrolytes produces dehydration and decrease of viscosity.

The strong hydration of ekhydrones prevents their being ultravisible. If, therefore, enhydrones of globulins, casein, yeast phosphoprotein, etc., are treated with increasing amounts of an acid or of a base, the oscillating particles, or aggregates of particles, that have been visible in the dark field, disappear, and the field gradually becomes optically void

If dried yeast, for instance, is treated with water at 37° C., a maceration product is obtained, which contains the protein in an extraordinarily fine dispersion. Even if the protein is now coagulated with highly dilute acid, it is no longer possible to restore the original high viscosity. If this coagu-

* These correspond to what Leonor Michaelis calls "lazy ions." See his paper in Vol. I of this series. *J. A.*

lum is rubbed with water there will result quite stable milky sols, but their degree of dispersion is much coarser than that of the original maceration product. This shows that other constituents of the yeast cell participate in producing this high degree of dispersion. Perhaps the lipoids of the cell might act in this way. This observation is of the greatest importance if we want to understand certain enzymatic actions that occur in the maceration product. As will shortly be pointed out, all other milieus than maceration fluid are considerably less favorable for the action of the peptide-splitting enzyme of the yeast cell, and also for that of seeds of leguminosae. This enzyme, once precipitated from the original milieu, does not show any such high activity as has been present in the maceration product. The way in which enzymes are dispersed in the cell colloids is, therefore, the most important preliminary condition for their activity. With the change in the colloidal state of these cell constituents, which from now on we will call *"enzyme colloids,"* enzyme action, which is controlled by them, is favored or hindered.

The proteins of the cell, according to our present knowledge, certainly occupy a very important place among enzyme colloids. The next section will show how the change in the peptide-splitting action of yeast maceration- and press-juices, as well as of ox pancreas, parallels the change in the degree of dispersion of their enzyme colloids, which in these cases is a phosphoprotein.

As regards the nature and functions of these enzyme colloids, their character is closely connected with that of enzyme action in general. On investigation of these substances there arises a whole series of questions that demand explanation. We must especially determine:

1. Whether individual enzyme actions, which are more or less specific, are dependent on specific enzyme colloids, or if they can be transferred from one colloidal substance to another. Willstätter and his co-workers [8] maintain that they are able to transfer invertin from one substance to another without changing the activity of the enzyme.[*] But as in spite of their endeavors and those of H. von Euler [9] invertin is still combined with foreign bodies which cannot be eliminated without entirely destroying the activity of the enzyme, we have no indication whether invertin in the purest preparations is combined with a specific enzyme colloid or not. The investigation of other enzymes by Willstatter has not advanced as far as that of invertin as regards the elimination of foreign substances. But these facts show that in this case also, the final separation of a true enzymatic substance from the cell colloids has not been accomplished. This raises the question:

2. Whether such a true enzymatic substance exists at all. The failure to separate such a substance from its ultimate carriers, which are represented by enzyme colloids, may be due to the fact that this substance is rapidly destroyed in an uncombined state. On the other hand, we still do not know any fact that would furnish proof for the existence of such a true enzymatic substance. Our knowledge of the nature of enzymes stops at the moment when we try to isolate this enzymatic substance by final adsorptive purification. The question still remains open whether or not enzyme action is due to certain colloids, which, though ineffective as mere materials in themselves, may nevertheless act as enzymes, if in a certain physico-chemical state, i.e., equipped

[8] Willstätter, R., see references in his paper in this volume; also Willstätter & Schneider, *Z. physiol. Chem.,* 142, 288 (1924); *ibid.,* 151, 1 (1925).
[*] See paper by R. Willstätter, this volume. *J. A.*
[9] Euler, H. von, "Chemie der Enzyme" (Munich, 1925); also Euler & Josephson, *Ber.,* 56, 446 and 453 (1923).

with certain energies from electric charges, or with still unknown ionic charges and their incidental concomitants (state of hydration, extension of surface).

It has been universally established that enzymes do not diffuse through membranes. But this fact does not show whether this is due to enzyme colloids or to the hypothetical pure enzymes, because these may themselves be colloids. But as it has not yet been possible to prepare pure enzymes, a further discussion of this subject is unnecessary.

3. If we should finally succeed in separating a "pure enzyme" from the enzyme colloids, we must then decide whether these colloids represent only incidental ("accessory") impurities, or if they possess a special biologically defined importance in enzyme action. The assumption of the preparative chemists, for instance, of Willstätter's school, that these substances are only unimportant impurities, is unacceptable, for it will be shown in the next section that these colloids influence and even determine the manner and the degree of enzyme action.

III. Isolation and Colloid-Chemical Analysis of Phosphoprotein, Acting as Enzyme-Colloid, Which Has Been Obtained from Maceration Juice of Yeast and from Pancreatic Press-Juice

E. Abderhalden and A. Fodor [10] were the first definitely to show that colloid substances participate in the action of enzymes in the milieu furnished by maceration and press-juices of cells and tissues. Further proof for this conception was furnished by A. Fodor [11] in his kinetic estimations and attempts to isolate the colloid. Enzyme studies by both authors showed changing kinetics of polypeptide splitting by yeast maceration juice, i.e., the time-curve of splitting changes materially according to the H-ion concentration of the milieu.

Further experiments by these investigators showed that the enzyme which splits polypeptides has no definite and unchangeable H-ion concentration optimum. Quite to the contrary, the optimum varies over a wide range, according to the chemical nature of the polypeptide substrate, and may also be considerably changed by the addition of neutral salts to the enzyme milieu. The following experiments refer to this question.

Among numerous experimental data we mention the splitting of glycyl-l-leucin, where transitions from one kinetic form into another are especially pronounced.

The authors demonstrated by preliminary experiments that no irreversible changes of the yeast juice enzyme occurred under the experimental conditions of temperature and H-ion concentration (or free alkalinity). Table I (see below) shows that the experiments were carried out at 25° C. and were continued for 50 minutes at the utmost. No enzyme destruction could possibly happen in so short a time, and we have to consider only those changes of the enzymatic substance produced by the alkalinity, the nature of which we shall discuss later on.

Most dipeptides, especially glycyl-l-leucin, show continuous transitions from the almost linear ratio

$$K = \frac{x}{t},$$

[10] Abderhalden, E and Fodor, A., *Kolloid-Z.*, **36**, 250 (1925); *Fermentforsch.*, **2**, 151 and 211 (1918).
[11] Fodor, A., *Kolloid-Z.*, 1925-6.

which occurs in acid solution (pH $= 6.27$-6.76) to the logarithmic curve

$$K = \frac{x}{t} \cdot \ln \frac{a}{a-x}$$

which occurs at about pH $= 7.37$. With increasing concentration of OH-ions this logarithmic curve changes more and more into parabolic curves, which almost conform to the equation

$$K = \frac{x}{\sqrt{t}}$$

Table I explains these relations.

The monomolecular time law is valid with glycyl-l-leucin at pH $= 7.37$ up to a transformation of about 66 per cent, which occurs in the first 40 minutes.

The tables and curves show that the time-course of the splitting of dipeptides is a function of H-ion concentration. This finding contradicts the statements of L. Michaelis and his co-workers who have stated that in numerous enzyme-actions the curve of time plotted against amount of change is independent of the acidity of the milieu.

The mathematical formulas of column 6, 7 and 8 of the table have been based on the adsorption isotherm

$$\frac{dx}{dt} = K\,(a-x)^n,$$

supposing the value of the exponent to vary from $n = \frac{1}{2}$ through $n = 1$, up to $n - 1.5$. Then the corresponding constants are:

$$K = \frac{1}{t}\left[\sqrt{a} - \sqrt{a-x}\right], \quad \text{and} \quad K = \frac{1}{t}\ln\frac{a}{a-x}, \quad \text{and} \quad K = \frac{1}{2t}\left(\frac{1}{\sqrt{a-x}} - \frac{1}{\sqrt{a}}\right).$$

We must now establish how the validity of the adsorption isotherm can be visualized, and what causes the variation in the value of the exponent.

The first point is most simply explained by supposing that the subtrate is absorbed by the colloidal constituent of the enzyme system. During this process the velocity of reaction at each instant is not in proportion to the substrate concentration $(a - x)$ as in a monomolecular reaction but to the nth power of this substrate concentration. In other words, the velocity of reaction is determined by $\frac{dx}{dt}$ which constantly adjusts itself to the adsorbing surface of the colloid and to the solution.

The second question involves the possibility of a continuous variation in the value of the exponent n. We should like to point out that experimental data of Abderhalden and Fodor, as well as of A. Fodor and B. Schoenfeld show that the adsorption of amino-acids, polypeptides, carbohydrates, oxyacids, etc., by animal charcoal under ordinary experimental conditions, may in fact be represented by equations of the general form

$$x = K\,(a-x)^n.$$

In these equations the exponent n may vary from a true fraction to unity, according to the relation between the amount of adsorbent and the concentration of the adsorbed substances. If there is a great deal of free surface present compared to the amount of adsorbed substance, n may become $= 1$, while the opposite ratio results in making n fractional.

TABLE I. *Splitting of Glycyl-l-Leucin.*
$a = 0.0625$ in 5 ccm. of reaction mixture.

1	2 Time in Minutes	3 Xg pro 5 ccm.	4 $K=\dfrac{x}{t}\cdot 10^4$	5 $K=\dfrac{x}{\sqrt{t}}\cdot 10^4$	6 $K=\dfrac{1}{t}\log\dfrac{a}{a-x}\cdot 10^4$	7 $K=\dfrac{1}{t}[\sqrt{a}-\sqrt{a-x}]\,10^4$	8 $K=\dfrac{1}{2t}\left[\dfrac{1}{\sqrt{a-x}}-\dfrac{1}{\sqrt{a}}\right]$	9 pH
1	10	0.0105	10.5	....	183.9	0.22	....	6.27
	20	0.0224	11.2	....	221.9	0.25	....	
	30	0.0299	9.9	....	216.9	0.23	....	
	40	0.0380	9.5	....	234.2	0.23	....	
2	10	0.0122	12.2	20.6	217.1	0.26	....	6.76
	20	0.0220	11.0	26.1	216.9	0.25	....	
	30	0.0305	10.2	29.6	223.2	0.24	....	
	40	0.0384	9.8	32.2	238.2	0.24	....	
3	10	0.0152	15.2	25.6	278.6	0.32	0.0598	7.37
	20	0.0258	12.9	30.6	256.1	0.29	0.0610	
	30	0.0352	11.7	34.1	276.1	0.28	0.0684	
	40	0.0416	10.4	34.9	273.4	0.27	0.0729	
4	10	0.0216	21.6	36.4	423.9	....	0.0945	8.28
	20	0.0357	17.8	42.5	423.3	....	0.105	
	30	0.0440	14.7	42.7	405.8	....	0.1117	
	40	0.0493	12.6	41.3	388.7	....	0.1176	
5	10	0.0235	....	39.5	471.6	....	0.1064	8.41
	20	0.0372	....	44.3	401.1	....	0.1244	
	30	0.0445	....	43.2	414.9	....	0.1151	
	40	0.0493	....	41.2	388.7	....	0.1176	
6	10	0.0177	....	29.7	332.9	....	0.0725	8.81
	20	0.0297	....	35.2	322.4	....	0.0766	
	30	0.0308	....	35.7	296.2	....	0.0746	
	40	0.0414	....	34.7	274.4	....	0.0721	
7	10	0.0116	....	36.7	205.3	....	....	9.33
	20	0.0184	....	41.2	174.3	....	....	
	30	0.0254	....	24.6	173.8	....	....	
	40	....	....	....	....	....	....	

When we consider the shape of the curves of time plotted against amount of change that results when polypeptides are split by fresh macerated yeast juice in the case where $n < 1$ the curves approximate straight lines, which give the impression that the velocity of amount of change is independent of time. S. Arrhenius [12] states that curves of the simplest enzymatic reactions, i.e., of those which are not complicated by any secondary conditions, correspond to a reaction of zero order and are, therefore, represented by a straight line. But this view is contradicted by the fact that the same system shows convex curves at an H-ion concentration which insures a greater velocity of reaction. If $n = 1$, this curve is perfectly logarithmic, if $n > 1$, it assumes a pronounced parabolic shape.

If in these systems too the ratio between adsorbing surface and substrate (adsorbate) concentration is supposed to cause the change in the value of the exponent, the transition of the exponent n from a true fraction to unity may be explained by the fact that with optimal concentration the surface increases materially. The degree of dispersion of the colloidal constituent of the enzyme system has been increased. As we shall presently show, such an increased degree of dispersion has actually been established by A. Fodor. This fact supports the view that enzyme action in the case of polypeptide splitting, involves a poly-disperse "enzyme-colloid" as active agent.

We now come to the question what causes this apparent adsorption isotherm. It might be suspected from the outset that a colloidal constituent of the enzyme system participates in the appearance of the adsorption isotherm, and this has been actually established experimentally. But how can this colloid be imagined to function? Omitting for the present the place this colloid occupies in the enzyme system, we may imagine that this colloidal substance adsorbs the substrate, which is then split. But the speed of reaction, if the rate of splitting is very high compared with the rate of diffusion of substrate towards the colloidal surface, is dependent on the adsorption equilibrium, which varies with the concentration of the gradually disappearing substrate. The velocity of splitting, therefore, is governed by the velocity of diffusion. If the constant of the velocity of diffusion is K, the velocity of splitting must be $\dfrac{dx}{dt} = K(a - x)^{\frac{1}{n}}$. In other words, the velocity of reaction is not in proportion to the amount present $(a - x)$ as in an ordinary molecular process, but is—at least generally—proportional to the $\dfrac{1}{n}$th power of this amount. However, if we are dealing with surfaces that are relatively large as compared with the amount of substrate, the velocity of reaction becomes proportional to the amount still present, i.e., $\dfrac{1}{n} = 1$. By the splitting of the adsorbed amount $x = K(a - x)^{\frac{1}{n}}$ at the surface, the adsorption equilibrium is disturbed and adjusts itself again. The exponent n is supposed to remain constant up to about a 60 per cent change in the substrate, which is not unreasonable.

But the change in kinetics may also be explained in another way. The

[12] S. Arrhenius, *Medd. K Vetesk. Nobelinstitut*, **3**, No. 13 (1916); also "Quantitative Laws in Biological Chemistry, London, 1915.

substrate may not act as an adsorbent, but may *eluate* * a certain constituent of the enzymatic system, which is adsorptively combined with our colloid. We shall discuss the nature of such an eluable constituent in the next chapter. There we shall designate it as *"zymohaptic substance."* It is absorptively combined with the enzyme colloid. In this elution, the substrate which acts as eluent is supposed to be split in proportion to the eluated amount of zymohaptic substance. It is easy to understand that here similar changes in the curves are produced as in the first case; for it is obviously unimportant for the final picture of the splitting process whether the adsorbed substrate is split at the colloidal surface itself or if the splitting takes place after complete elution, providing that the velocity of elution and hydrolytic action are relatively high compared with the velocity of diffusion of the substrate toward the colloidal surface. If the effective surface of the enzyme colloid is relatively small, elution by the substrate, which is present in excess, will proceed proportionately, so that the same change occurs over equal periods of time. But if the degree of dispersion of the colloid increases, we find a relatively higher adsorption initially than later on. The curves of time and amount of change, therefore, assume a convex shape. Table II shows that this occurs when the alkalinity is increased. At optimal H-ion concentration there occurs an almost explosive initial splitting, which later on decreases more sharply in proportion to the amount of substrate which has been initially split.

The next chapter will show that the latest experimental results support this theory of elution. Adsorption of substrate by enzyme colloid precedes elution.

But as soon as adsorption of substrate by the colloidal constituent of the enzyme system has to be taken into account, we are confronted by specific relations of individual substrates with colloidal surfaces. This adsorption may take place directly, or by removing an enzymatically active constituent from the colloidal surface (elution). This relationship may be a more or less intensive one, and *a priori,* the extent of substrate splitting may vary correspondingly. Yeast maceration juice actually splits certain polypeptides with great velocity, whereas with other polypeptides the reaction proceeds much more slowly.

But this relation is shown still more clearly by the position of optimal enzyme action in the scale of H-ion concentration. If optimal H-ion concentration were determined only by the state of enzymatic constituents, which has been produced by the influence of medium reaction, the optimum would always be at the same H-ion concentration. The same would be the case if solely the change of state of the substrate could influence activity. Experiments, however, have shown that the position of the optimum varies with the substrate, and also that it may be strongly influenced by numerous other factors, for instance by addition of salts. The first fact suggests the importance of the specific nature of the substrate. The susceptibility to addition of salts, however, shows that a variation of the state of enzyme constituents is also very important. Some experimental data are given in Table II.

The table shows that if the molecule of the polypeptide substrate increases, optimal H-ion concentration is shifted toward the acid side of the scale of H-ion concentration. Experiments with glycyl-aspartic-acid and with l-leucin-aspartic acid showed on the other hand that the optimal H-ion concentration of those substrates is likewise on the acid side of the scale. This seems to

* Elution is selective adsorption. See paper by R. Willstatter, this volume. *J. A.*

Table II.

Polypeptide, and Name of Author	pH	Cm.³ n/50 NaOH After			
		10 Min.	20 Min.	30 Min.	40 Min.
l-Leucyl-glycin .... [a]$_D^{20}$ = + 85.5° E. Fischer, *Ber.*, 39, 2893 (1906).	6.64 7.24 7.56 7.86	0.49 ... 0.53 0.47	0.55 0.73 1.08 0.84	... ...	0.99 1.18 1.26 1.01
l-Leucyl-glycyl-glycin .... [a]$_D^{20}$ = + 45.70° E. Abderhalden and A. Fodor, *Ber.*, 39, 561 (1906).	6.22 6.66 7.26 7.80 8.29	0.17 0.25 0.34 0.35 0.16	0.31 0.51 0.57 0.57 0.26	0.47 0.68 0.76 0.69 0.35	0.64 0.73 0.91 0.79 0.47
l-Leucyl-diglycyl-glycin .... [a]$_D^{20}$ = + 45.70° E. Fischer, *Ber.*, 39, 2893 (1906).	6.74 7.29 7.68	0.03 0.19 0.18	0.18 0.32 0.23	... ...	0.22 0.45 0.30
l-Leucyl-triglycyl-glycin ...... [a]$_D^{20}$ = + 28.14° E. Abderhalden and A. Fodor, *loc. cit.*	6.89 7.28 7.56 8.09 8.50	0.12 0.12 0.07 0.03 0.04	0.17 0.18 0.19 0.08 0.06	... 0.24 0.23 0.15 ...	0.27 0.34 0.27 ... ...
l-Leucyl-pentaglycyl-glycin .... [a]$_D^{20}$ = + 5.94° E. Abderhalden and A. Fodor, *loc. cit.*	6.16 6.64 7.33	0.00 0.23 0.12	... 0.28 0.19	... ...	0.16 0.31 0.24

suggest that the electro-chemical nature of the substrate is highly important, and that peptides, which are strongly dissociated in faintly acid solution may also be split most easily in such solution. The substrate (its salt, of course) must therefore be electrolytically dissociated to a certain extent in order to show optimal activity.

As to change of optimum upon addition of neutral salts to these enzymatic systems, experiments show that the optimum may be shifted as far as pH = 6.35.

These facts show that the optimum of peptide-splitting by yeast maceration juices has no definite position, but is variable. The optimal H-ion concentration is determined both by the state of dissociation and by the physical state of the enzyme system. The outside limit of this optimum is about pH = 8.5. At a higher pH we find usually strong initial activity, which soon decreases. We shall see that this decrease is closely connected with the colloidal state of a protein which must be considered as an enzyme colloid. In the next chapter it will be shown, that even under conditions, where this state can no longer have any influence, an optimum establishes itself at a similar place of the scale of H-ion concentration. The state of dissociation of the substrate itself must, therefore, be important.

After establishing by kinetic measurements as well as by fixing the optimum of activity, that colloidal substances participate in the splitting of polypeptides in the enzyme system, experiments to identify and to study those colloids could be outlined.

Yeast extract (press or maceration-juice) represents a mixture containing several colloidal substances. Some of them, so-called yeast-gum for

instance, are classified as carbohydrates. We also find several proteins. These two groups of substances can be separated from each other by coagulating part of the proteins from the extract by dilute acid. The remainder may be precipitated by salt solutions. Yeast-gum remains in the filtrate from the last precipitation, together with other noncolloids.

Upon coagulation of proteins by acid, the solution loses its characteristic appearance in the ultramicroscope. The Tyndall phenomenon, which is prominent in yeast maceration juice, likewise decreases. The original picture in the ultramicroscope shows a large number of particles with active Brownian movement, but after precipitation by acid the field is practically void.

The nature of protein coagulated by acid has been thoroughly studied, and a remarkable phenomenon was observed. The physical behavior of the first fractions of this coagulation is quite different from that of the subsequent fractions, though the chemical composition shows no appreciable difference. We find 14.83 — 14.98% N and 1.58 — 1.73% P. From its behavior it is a phosphoprotein, for which the name *yeast phosphoprotein* is proposed.

The physical behavior of the fraction, obtained by coagulating dilute yeast maceration juice with so small an amount acid that the H^+ of the solution is less than 10^{-4} is as follows:

It has such finely dispersed particles, that a milky opalescent colloidal sol of relatively high stability results upon merely rubbing with water, i.e., without any added peptizer. The ultramicroscopic picture of this sol corresponds almost exactly to that of the original yeast maceration juice from which it has been obtained.

From the nature of the protein, the sol particles are negatively charged enhydrones, that migrate to the anode. They may be coagulated by cautious addition of acid to the sol. Coagulation takes place at pH = 5.10. If alkali, however, is added to the sol, we get the characteristic swollen solution of alkali-protein (ekhydrones), which appears transparent and shows an ultramicroscopically empty field. If acid is added to these alkaline solutions, optimal coagulation occurs at pH = 4.5. The amount of salt which is formed during neutralization of the base, therefore, changes the coagulation optimum. If the protein is allowed to stand alkaline for some time, phosphoric acid is split off and a new protein derivative results with a coagulation optimum at pH = 6.6.

Those fractions of the phosphoprotein that have been coagulated from yeast maceration juice with large amounts of acid are, in contradistinction to the before-mentioned fractions, coarsely dispersed, do not form sols, and dissolve only in dilute alkali. Thus swollen alkali protein is formed, which has the same coagulation optimum by acid of pH = 6.6.

This colloid-chemical analysis shows that the mode of coagulation determines the physical properties of our phosphoprotein. The finely dispersed fraction of the phosphoprotein is the only protein body that we know of, which forms directly stable colloidal sols with water, instead of hydrated solutions, as do native albumin or gelatin. Neither casein nor any of the known varieties of globulins show this peculiarity.

Many fractions and filtrates have been systematically examined for their enzymatic activity towards polypeptides. Only the first acid coagulates, however, were active, i.e., those that form spontaneously a typically colloidal sol with water. The activity of these sols was certainly much less intense than that of the original maceration juice, but still strong enough to permit a dis-

tinct estimation of the splitting action. The following facts show not only that certain fractions participate in this activity, i.e., the very first fractions yielded by acid coagulation, but that the degree of dispersion of the coagulated phosphoprotein itself must be considered. If all coagulable substances be coagulated by acid at the same time, the coagulate does not form stable sols and shows no activity at all. The conclusion, therefore, is justified, that activity depends *a priori* on the finely dispersed state of the protein coagulate. It is strange to say that active systems either are not formed by agents that increase dispersion of coarsely dispersed coagulates which in the ultramicroscopic field show large aggregates without Brownian motion.

If active sols are treated with alkali, the fermentative activity increases up to a point where hydration of protein begins to counteract this effect. Protein ekhydrones are inactive. The optimum of enzymatic activity, therefore, is to be found in these systems at a certain degree of hydration. Addition of alkaline phosphates is more favorable, because they produce less hydration at the same alkalinity. If neutral salts are added simultaneously with the alkali, the optimum is also shifted to numerically higher values. Small differences in the fineness of dispersion also influence the position of optimal H-ion concentration.

To summarize, we are justified in drawing the following conclusions from these experiments:

1. The substance in yeast maceration juices active against polypeptides may be transferred from the milieu by a phosphoprotein.

2. Only the highly dispersed fractions of this protein are active. If no highly dispersed fraction can be obtained from a species of yeast, the activity of the coagulate is zero.

3. There exists a marked parallelism between the degree of dispersion of the active sol as observed ultramicroscopically, and enzymatic activity.

4. Only enhydrones, or alkali-enhydrones are active; the strongly hydrated ekhydrones are inactive.

5. Optimal H-ion concentration depends on a definite state of hydration of the protein carrier of enzymatic activity.

These facts show that yeast phosphoprotein may act as a colloid in an enzyme system. In this case intensity of activity depends unquestionably on the physical state of the colloid. The designation "enzyme-colloid" has just been suggested for such a colloid. What does it really do? Does it carry the active enzyme in the original maceration juice milieu, or is it only an accessory substance? There are two possibilities if it carries the active enzyme: an enzymatically active substance may exist which occurs together with the protein in the maceration juice and *ceteris paribus* in the original cell milieu too. On the other hand, it may be that no such substance exists, and that enzymatic activity is an essential property of the phosphoprotein or of one of its fractions. The intensity of action depends on the rate of dispersion, which is highest in the maceration juice. This high degree of dispersion cannot be restored to the protein, once it has been coagulated.*

But if the colloid is only an accessory substance, this would mean that it has no real importance at all. In this case the real, i.e., specifically active enzymatic substance, is simply accidentally carried down during the coagulation of the colloid.

* The possibility of cumulative protection should be considered here. See Vol. I of this series. *J. A.*

The accessory character of the protein, however, is disproved by the fact that pancreatic press juice contains a phosphoprotein of almost the same chemical composition. This protein may be fractioned by acid coagulation in exactly the same manner as that from yeast. Only the most highly dispersed fractions of this protein form sols and are active. Later experiments with seeds of leguminosae showed that with them, too, active protein may be coagulated by acid from maceration juices of pea meal, but also with a strong decrease in activity. All these results suggest the importance of cell proteins in enzymatic processes. A combination of these proteins with certain unknown, possibly hypothetical, specific enzymatic substances (called *zymohaptic* substances in the next section) is supposed to exist in the cell. In the next chapter further proof will be furnished for this view. We shall investigate if and to what extent these zymohaptic substances can exist as independently active complexes of enzymatic systems apart from the enzyme colloid. Perhaps we shall succeed in defining more closely the conception of enzyme colloid.

IV. Cleavage of the Enzyme-Colloid System by Kaolin

The fact that cellular proteins are carriers of enzymatic action and that their state and colloidal dispersion determine the intensity of this action, justifies us in calling them *enzyme colloids*. Such a terminology does not *a priori* clearly define the nature of enzyme systems, i.e., we have besides certain activating constituents, carried by the enzyme colloids. Such a constituent might be a certain chemical group of the enzyme colloid itself, or else a separate specific substance that combines with the colloid (perhaps by adsorption). It is also possible that several different substances combine with the enzyme colloid; there may be a third substance, for instance, through which the specific activating substance is adsorbed by the enzyme colloid, etc. All these substances together constitute the enzyme system.

The specificity of the individual constituents may be absolutely different, and the enzyme colloid, therefore, may be non-specific and exchangeable. In this case it should be possible to transfer the really active substance from this colloid to another. Willstatter * and his coworkers found a similar case in invertin. Their method of purifying enzymes by different adsorbents is based on this fact. As kaolin, aluminium hydroxide, etc., do not absorb the colloidal carriers of the real enzymatic substances to the same degree as they adsorb the active substances, the latter can be separated from the colloids. But according to Willstätter this separation does not always succeed. Plant lipases, for instance, could not be separated from protein, and it is possible that this colloid is identical with the enzymatic substance.

Other possibilities must also be considered. Let us suppose that with a certain enzyme a specific active substance actually exists, which may be called *"zymohaptic substance"* (*zy. s.*). This active substance may be combined with a certain amount of colloidal carrier (*en. coll.*). This type may be expressed by *en. coll.-zy. s.* The ratio between the two is fixed by the fixing power of the enzyme. Now it is quite possible that this combination is distributed at the surface of a larger mass of the colloidal *en. coll.* involved. This distribution could, however, occur at the surface of a second colloid.

For the present we can only state that it has not been possible to separate

* See paper by Richard Willstatter, this volume. *J. A.*

and prepare a pure active substance such as we suppose our *zy. s.* to be, despite the efforts of such investigators as Willstätter, H. v. Euler and their numerous coworkers. All attempts to prepare such a substance free from all traces of colloids were defeated by the fact that the activity disappeared during the attempts at purification. Obviously colloids are indispensable for the conservation of the *zy. s.* But the existence of such a *zy. s.* is purely hypothetical. This is shown by our discussion. If activity demands the presence of accompanying colloids as *conditio sine qua non,* it is impossible to decide whether continuous attempts to purify these colloids actually destroy a hypothetical *zy. s.,* or if this loss of activity is caused by another still unknown process, apart from chemical destruction.*

We shall continue to assume the existence of a hypothetical *zy. s.,* but only for expediency and in order to make our views more easily intelligible. We make no claim as to the real existence or non-existence of a *zy. s.* Purely as a working hypothesis, we assume a combination *en. coll.-zy. s.* exists in yeast maceration juices, in pancreas juice, and in seeds of leguminosæ, and that the above mentioned protein substances occur as *en. coll.* Experiments show that these proteins may indeed be easily precipitated from such maceration juices in an active state.

Attempts were made to split this combination by various methods.

If maceration juices from dried yeast are treated with kaolin, the most diverse changes in the original activity may be observed according to experimental conditions. We estimate the amount of a polypeptide or peptone split under exactly the same conditions, taking what occurs during the first two hours of action. We, therefore, examine the initial velocity of reaction under uniform conditions.

Sometimes the filtrate from the kaolin adsorbent is free from protein but still active; on the other hand it may be inactive. But these contrary results are not produced merely by variation of the amount of kaolin, for they also appear when the same quantity of adsorbent is used, the most important factor being the manner in which the maceration juice is treated with kaolin, i.e., whether it is all added at once or in many small portions.

The activity of the kaolin adsorbate may also vary considerably, for it may take up not only proteins but also active material either in whole or in part. In the first instance the kaolin adsorbent shows a high activity, in the other instance a very low one in its direct action on substrates under like conditions. Another variable property of adsorbents is their eluative power. The active phosphoprotein coagulum which is obtained directly from maceration juices by cautious acid coagulation, may be eluated even by means of glycocoll solutions or silk peptone. After treatment at 37° C., a filtrate is obtained which is free from the major portion of protein, and which at the same time shows activity.

This separation from the major portion of protein may be accomplished still more successfully by eluating the above mentioned kaolin adsorbent with glycocoll or silk peptone. But in this case also the results of the treatment are quite variable. Sometimes a highly active eluate may be prepared from the kaolin adsorbent at 37° C., sometimes the effect is very slight. This result too depends on the manner in which kaolin is used.

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* It is quite possible that the activity is due to some certain orientation of certain molecules in the surface of the enzyme particle; and it is quite conceivable that these "active" molecules may be an integral part of the "enzyme colloid" or of an incidental "impurity." *J. A.*

If protein-free filtrates of kaolin adsorbate are treated with fresh amounts of kaolin or with aluminium hydroxide in order to transfer their activity to these adsorbents, the treatment is quite successful. If one works cautiously and quickly, it is even possible to transfer the total activity of these fluids to the surface of the adsorbent, where it may be found again almost undiminished in quantity. But it is impossible to remove activity from these adsorbents by means of any eluent. The same thing happens if active eluates of the original kaolin adsorbates, which contain proteins in too small an amount to be demonstrated by the usual methods, are treated again with kaolin or aluminium hydroxide. By working quickly, activity may be transferred from the eluates to the surface of the adsorbent without any appreciable loss, but then elution fails under all conditions.

This shows that a considerable difference exists between cases where the adsorbent takes up activity together with large amounts of protein, and other cases where the protein is lacking. Other interesting results are obtained from experiments on stability of different fluids and adsorbates. The stability of original yeast maceration juices has heretofore been studied. Fresh extracts show variations, activity increases as well as decreases, but generally it is quite stable, at least if the extracts are handled carefully.

However in filtrates from kaolin adsorbates, which are poor in protein, activity is much less stable. Glycocoll eluates, if not dialyzed, behave in the same way. But if they are dialyzed they may be separated from glycocoll as well as from other substances without any material decrease in activity even after a dialysis of 6-8 days. The stability of kaolin adsorbates decreases also, especially of those which have been obtained from filtrates or eluates without detectable protein content.

If we consider maceration juices of seeds of peas or beans, which are almost as active and as stable as those of yeast, we have to deal with different conditions. The same apparent irregularities are found when the activity ratio is balanced between adsorbate + filtrate and original maceration fluid, whose activity may even be totally transferred to the surface of kaolin. It may also happen, as with yeast, that the filtrate contains only traces of protein, but is quite active. This shows that with leguminosæ, too, the real carriers of enzymatic activity may be separated from the main mass of protein.

It must be emphasized, however, that neither with yeast nor with leguminosæ, can the active and apparently protein-free fluids be called positively "protein-free," even if the most sensitive protein tests are negative. For it has been established that the presence of phosphates as well as of the products of protein decomposition (for instance amino-acids) inhibits the Spiegler or Esbach reaction.

The difference between leguminosae and yeast consists in the fact that there is no way to obtain active elutions from the kaolin adsorbate of leguminosae, without a considerable protein content, while this has been possible when working with yeast. Glycocoll and peptones, as well as many active alkaline and acid elution reagents, failed absolutely. The adsorbate is quite active, but in contradistinction to yeast adsorbates, the activity cannot be eluated.

Apart from this, experiments on stability show similar results to those carried out with yeast. Stability is highest in the maceration fluid; filtrates that are poor in protein and adsorbates are much less stable. At the same time it has been observed that in leguminosae the hypothetical carriers of

activity, i.e., our *zy. s.*, are so strongly combined with final traces of protein, that separation of all protein causes loss of activity. This is shown by the behavior of the filtrate from the kaolin adsorbate. This filtrate cannot be separated from the last traces of protein without becoming inactive. If this fluid is dialyzed, the protein is coagulated as a very fine almost unweighable turbidity and the filtrate becomes inactive. (The fluid that has been dialyzed off does not participate in this inactivation.) In this instance too it is possible that the *zy. s.* is immediately destroyed by the removal of the last trace of protein, which exercises the protective effect of the enzyme colloid. On the other hand colloid and *zy. s.* may perhaps form a single unit, and a separation is impossible.

A theoretical discussion of the causes of these apparent irregularities in the isolation of activity, will incidentally furnish a coherent explanation of most of the experimental facts, which at first glance seem rather confusing in their variety.

A preliminary experiment will be aidful. If maceration juices from yeast or leguminosae are treated successively with increasing amounts of kaolin, the following results are obtained:

If the amount of kaolin is sufficiently small we find, paradoxically, that in spite of the removal of activity by the adsorbent, the filtrate from the kaolin adsorbate is just as active as the original maceration juice. There must have been formed some equivalent which replaces the activity removed. If the amount of kaolin is increased, this development of activity soon stops, and more or less considerable losses of activity are found compared with the original maceration juice.

Special experiments showed that this increase in total activity is not produced by the removal of inhibiting substances by kaolin. In these experiments the systems (maceration juice alone), (maceration juice + kaolin), and (filtrate from maceration juice + kaolin) have been investigated. It has been shown that under suitable conditions, the activity of the system (kaolin + maceration juice) remains unchanged in the filtrate after the removal of kaolin. This clearly contradicts the assumption of inhibiting substances, the removal of which by kaolin would produce increase of activity.

This temporary increase in activity by kaolin can be explained only by the assumption that kaolin facilitates the separation of our hypothetical carrier of activity, the *zy.s.*, from the original milieu in which it exists in the maceration juice, and that the substrate helps in this separation. But, according to all that we have learned so far, this milieu is represented by the major portion of certain proteins that act as enzyme colloids. We, therefore, are dealing with the colloidal combination:

$$en.coll. - zy.s.$$
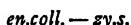
If we add kaolin (*ka*) to this system, the whole enzyme system is at first adsorbed by the surface of kaolin forming:

$$Ka - en.coll. - zy.s. \quad ,$$

But this system is relatively very labile. The last mentioned experiment shows that this adsorbate, under certain conditions, may be more active than the system *en.coll.-zy.s.* in solution! Kaolin, therefore, sensitizes this combination, which facilitates the action of the substrate. We shall now discuss how this comes about.

The adsorbate *ka.-en.coll.-zy.s.* is not only more sensitive towards the substrate than is *en.coll.-zy.s.*, but also towards larger amounts of kaolin. If we add kaolin to the suspension of the adsorbate, the following reaction takes place.

$$Ka. - en.coll. - zy.s. + Ka. = Ka. - en.coll. + Ka. - zy.s.$$
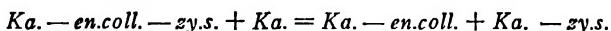
Gradually the first adsorbate is split and another, the so-called "second adsorbate," is formed. In this form of adsorption our *zy.s.* is apparently no longer combined with the main mass of enzyme colloid, i.e., the protein, but with the adsorbent kaolin. Consequently total activity decreases; for activity, or if we may so put it, its hypothetical carrier, *zy.s.*, is very unstable and rapidly destroyed when separated from protein.

This explains the apparent irregularity in the yield of activity when maceration juices are treated with kaolin. If the treatment is carried out under conditions which favor transference of the combination *en.coll.-zy.s.* to the surface of kaolin, we find high yields of activity after elution of the adsorbate by glycocoll. But we get low yields if a splitting occurs by treatment with kaolin and "second adsorbate" is formed. In order to secure high yields, the combination *en.coll.-zy.s.* should be transferred suddenly from the maceration juice to the surface of kaolin by shaking once with the optimal amount of kaolin. Adding this quantity of kaolin in many small portions causes low yields.

We may understand the cause of these differences if we recall the discussion in Section 1, which showed that adsorption always takes place at the whole surface, where the adsorbed substance is evenly distributed. In our case the real adsorbed substance is protein, which protects the *zy.s.* But if protein is combined with the surface of kaolin, fresh kaolin may act directly on *zy.s.*, all the more easily as the *zy.s.* is now in a sensitized state. This sensitization shows the specifically colloidal character of the reaction, and enables us to explain the nature of the elution process.

Owing to adsorption by kaolin, the combination between *en.coll.* and *zy.s.* is weakened, and our hypothetical *zy.s.* is liberated. This may manifest itself in two ways: *First*, either glycocoll or peptone (or polypeptide, etc.) which acts as "eluent," combined with the *zy.s.*, as has been assumed for invertin and its substrates by the school of Willstätter; even the affinity coefficient between the two substances has been estimated in this instance. As a *second* possibility, glycocoll displaces *zy.s.* according to the ratio of its own combination with *en.coll.*, i.e., in this case with the protein that participates in the reaction. Our latest experiments seem to support the second explanation, i.e., displacement.

This conclusion is based on the following facts: glycocoll does not influence the activity of the eluate at all. It may be dialyzed off without activity undergoing any change. Now if glycocoll should combine with *zy.s.*, we would expect it also to effect elution from those "second adsorbates" in which *zy.s.* is directly combined with kaolin. We know that this does not occur. On the other hand *zy.s.* must be very loosely combined with kaolin in this form, for the whole activity of the glycocoll eluate is easily recovered from kaolin adsorbate after adsorption. This would be impossible if *zy.s.* were closely combined with kaolin.

But the view that the elution reagent displaces *zy.s.* is supported by more convincing kinetic experiments which have been carried out with these ad-

sorbates and other fluids. The reaction may be represented diagrammatically thus:

$$Ka - en.coll. - zy.s. + glycocoll = Ka - en.coll. - glycocoll + zy.s.$$
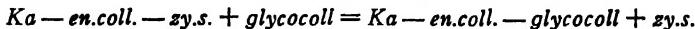
As above mentioned, the liberated *zy.s.* is wide open to destructive influences in this case, if these are not removed at once by efficient dialysis.

Kinetic measurements show the remarkable result that curves of time plotted against change of kaolin adsorbates are of zero order, i.e., straight lines. We remember from Section III of this paper, that in yeast maceration juices this takes place only at certain H-ion concentrations. In these instances the state of the enzyme colloid is much coarser, because it approaches the coagulation optimum. In contradistinction to the slow splitting produced by this state, favorable H-ion concentrations induce a rapid reaction with a logarithmic or parabolic curve of time plotted against amount of change.

This leads to the essential point of the proof: Substrates "eluate" *zy.s.* by removing it from its combination with *en.coll.*, being themselves adsorbed. Expressed diagrammatically:

$$en.coll. - zy.s. + substrate = en.coll. - substrate + zy.s.$$
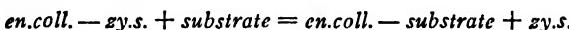
Displacement by a *certain fraction* of substrate, therefore, activates the *zy.s.* This displaced amount depends on two conditions: (1) on the adsorbing surface of enzyme colloid and (2) on the specific adsorptive power of the substrate.

The adsorbing surface is largest and most active in maceration juices with favorable H-ion concentrations. In this instance phosphoprotein shows highest dispersion and lowest hydration (enhydronic form). This state is most easily obtained in weakly alkaline natural yeast maceration juices, where adsorption occurs so rapidly that displacement elution which is caused by it, occurs under certain conditions more quickly than hydrolysis of the substrate by free *zy.s.* In kinetic estimations, excess amounts of free enzyme may be assumed in these cases.

Different results appear in all instances where surface conditions are unfavorable, as in maceration juices with unfavorable H-ion concentration or in kaolin adsorbates. Only slow displacement elution can occur in this case. Consequently an excess of substrate will be present, and the course of enzymatic splitting will make it appear that we are dealing with constant amounts of enzyme. By reason of this we shall find a more or less linear curve of time plotted against amount of change.

According to what has been mentioned under (2) above, substrates, or in general, substances that are incapable of adsorption, should not either eluate or be split themselves. This fact may be able to explain some experimental observations.

Amino-acids actually exist whose solutions do not possess the power of glycocoll; for instance d-*l*-leucin. Power to eluate, therefore, is certainly not a property of glycocoll as a cleavage product, but a specific property. (According to L. Michaelis,[18] and Willstätter[14] and his coworkers, invertin combines not only with the substrate but also with the cleavage products.)

[18] Michaelis, I., *Biochem. Z.*, 7, 488 (1907); *ibid.*, 49, 33 (1913); *ibid.*, 50, 50 (1914).
[14] Willstätter, R., see his paper in this volume. *J. A.*

Now there exist polypeptides which cannot be split, as we long know from the work of E. Fischer and E. Abderhalden; but this fact could not be explained. It should also be remembered that direct adsorption of amino-acids and polypeptides by yeast maceration juices has already been directly demonstrated by ultrafiltration methods. The question in what way "second adsorbates" of the type *ka.-zy.s.* can be active in spite of their showing no displacement elution of their activity with glycocoll or peptone, may be explained thus: Displacement elution is impossible, because, as is well known, kaolin will not adsorb amino-acids and peptones. But we still face the possibility that substrate and *zy.s.* react with each other on the surface of kaolin itself. Kinetic measurements actually show that splitting by the system *ka.-zy.s.* is of the same order of magnitude as in the filtrate of the first adsorbate where *zy.s.* is supposed to exist in a quasi free state.

We shall take up this special question: Is it at all reasonable to assume such a liberation of *zy.s.*?

Here we may consider the results with seeds of leguminosae above referred to, according to which such maceration juices, when treated with kaolin, do not produce adsorbates that can be eluated with glycocoll, peptone, etc. This may be due to several causes. Shaking maceration juice with kaolin may produce only "second adsorbates" of the type *ka.-zy.s.*, if the lability of the system *en.coll.-zy.s.* in the maceration juice is so much increased by kaolin, that it decomposes immediately. Under these conditions, part of the kaolin is charged with protein (= *en.coll.*) and another part with *zy.s.*

On the other hand the stability of the system *en.coll.-zy.s.* might be so high that it cannot be separated, at least not by means of the elution reagents which work well with yeast. Finally the proteins which act here as enzyme colloids may lack the power to adsorb considerable amounts of glycocoll or peptone and thus effect displacement.

Which of these three possibilities best represents the facts, has not yet been established and the question is still unsolved. But this is true: it is even more impossible to separate the carrier of activity (our hypothetical *zy.s.*) from the last traces of protein, than when working with products of yeast. If we try to remove the last traces of protein from the filtrate of the first kaolin adsorbate by operations like dialysis, hydrolysis by pepsin, or further amounts of kaolin, all activity vanishes. It seems that with yeast very small amounts of protein, that can hardly be detected by sensitive protein tests, cling to the active filtrate; but not in such an obvious form as with leguminosæ. When dialyzed, for instance, they show no coagulation of a protein character, as do leguminosæ products.

When we try to explain the nature of our *zy.s.*, our knowledge fails. We are dealing with a system that eludes further analysis, and which, therefore, must keep its hypothetical character for the present. This constitutes, therefore, the chief problem of enzyme research [16] today.

Conclusions

As far as we can learn from the behavior of polypeptide-splitting extracts, we may draw the following conclusions as regards the nature of the enzymatic systems in question: In the living cell the purely hypothetical carrier of activity is combined with large masses of protein, that are transferable to

[16] Fodor, A., *loc. cit.*

maceration and press juices. The intensity of activity depends on the colloidal state of these protein masses. With great losses in activity, certain substances that obviously participate directly in enzyme action, may be artificially separated from the major portion of the protein. But after this separation their activity is relatively rapidly destroyed.

The mechanism of substrate splitting is that the substrate itself displaces the active constituent in the protein mass by having a fraction of its total amount adsorbed by protein surfaces. . In this way substrates, so to say, themselves liberate or produce the amount of enzyme necessary for their splitting. In the living milieu, therefore, there will obviously be. present only as much active enzyme in a free state as corresponds to the actual substrate concentration.

The main point is that the active substance, whose activity depends greatly on the change of milieu, and which has been separated from the major portion of the protein, contains colloidal constituents, which cannot be removed without loss of activity. That is, enzymic activity involves the presence of colloids, and the question is what relation these residual colloids bear to the whole protein mass, i.e., to the enzyme colloid itself. Are they identical with the enzyme colloid and do they form perhaps a special fraction of it? Maybe a fraction with a special surface structure? All these questions are still unanswered at present. It is not impossible that the main mass of enzyme colloid is of a variable chemical composition, but consists of several fractions.* These fractions differ in their ratio of dispersion, and the highly dispersed fractions participate directly in activity. Perhaps the affinity of these fractions is strongest in respect to the hypothetical active substance and follows it through all phases. But the question whether such a substance really exists is still entirely hypothetical. There is as yet no proof to controvert the assumption that the most highly dispersed fractions of the enzyme colloids, owing to a still unknown specific structure, are themselves the carriers of activity.*

What may we imagine such a specific structure to be?

Let us remember that enzymatic activity, as far as hydrolytic actions are concerned, consists in the transference of the elements of water (II and OH) to the molecule of the substrate.[16] The chief function of enzymatic systems consists, therefore, to activate the inert molecule of water by making it labile. Different methods of this activation may be imagined. In ordinary acids, the solutions of which can also produce hydrolytic splitting, obviously the ions of the acid itself serve as activators of water. But there is no doubt that certain substances in the colloidal state can also effect an activation of this kind, by being hydrated in a certain form.

Owing to this hydration, they partially split the inert water molecule at their surface and labilize it. Living substance that is permeated by colloids, may contain numerous activators of this kind in its milieu. We do not deny that other substances may also participate in this activation of water by the colloids in question. But there can be no doubt that colloids are indispensable for enzymatic activity, and that enzymatic action ceases with their

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* Light is thrown on this point by the work of Edgard Zunz [*Arch. int. Physiologie*, 1, 427 (1904); *Bull. Soc. Roy. des sciences med. et nat.*, June 11, 1906], who showed that some albumose fractions are strong coagulants, while other fractions are powerful protectors. *J. A.*
* These most highly dispersed fractions might be in zone of maximum colloidality, where effective catalysis would be greatest owing to the balance between kinetic activity and free surface. See Vol. I, this series, Chapter I. *J. A.*
16 Fodor, A., *loc. cit.*; Bayliss, W. M., "The Nature of Enzyme Action."

disappearance from the enzyme system. This is the reason for the failure to accomplish a "final purification" of enzymes.

Let us imagine for a moment that our hypothetical *zy.s.* is actually a highly dispersed fraction of the enzyme colloid and is charged with active water molecules. In this case the added substrate will perhaps remove just this fraction from the total mass of the *en.coll.* because of its greater affinity of adsorption in respect to the coarser and less hydrated fractions. The fact that hydrated colloids show an especially small affinity of adsorption, is well known in the field of proteins (see Section II). In native maceration juices, however, the·amount of hydrated and active fraction is considerably higher, which produces the high activity of those extracts.

By acid coagulation and also by treatment with kaolin, a large percentage of these hydrated, highly dispersed fractions is aggregated. This decreases, and finally totally destroys, activity. Therefore, only a small portion of the highly dispersed fraction can be displaced (eluated).

But at this point research enters *terra incognita.*

Luciferin and Luciferase, the Luminescent Substances of Light-Giving Animals

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That life and the colloidal state are intimately connected is well known; that light and colloids are also related is not so generally recognized, although, with reference to the light of luminous animals, just as true. Nor is this merely because light production by living things is a "vital process," dependent on some obscure peculiarity of a vital mechanism. Light production is a purely chemical process. The substances concerned are in the colloidal state.

Among the forty different orders of living creatures which produce light, from the lowest (bacteria) to the highest (fish), the luminescent process appears essentially the same. An organic compound in the cells is oxidized. Oxygen and water are necessary. The light production is a chemi-luminescence analogous to that of phosphorus, or of pyrogallic acid when mixed with various oxidizing agents. Luminescence is quite independent of the living creature which produces the light. In fact the most convenient method of obtaining luminous material is quickly to dry a luminous animal. Its dried thoroughly powdered remains will luminesce again when moistened, and will serve as the raw material from which we may extract the luminescent substances. It is obvious that the problem of animal light is the nature of the oxidizable material and the oxidation process.

The most fundamental discovery in the field of bioluminescence is that of Dubois (1887), who demonstrated that two substances were necessary for light production. One, luciferin, is an oxidizable body, not destroyed by boiling its solution; the other, luciferase, is an enzyme concerned in the oxidation of the former with light production. Like other enzymes it is destroyed on boiling. Given these two substances in water solution containing dissolved oxygen, luminescence will occur. The living cell manufactures them. It remains for us to consider their chemical nature.

When luciferin oxidizes, no great chemical change appears to take place in the molecule. I have called the product, oxyluciferin, by analogy with the oxidation product of haemoglobin, oxyhaemoglobin. However, the mechanism of oxidation appears somewhat different from that of haemoglobin. As we shall see, it is more to be compared with the oxidation of a leuco-dye to the colored dye itself.

Luciferin solution is prepared by making a hot water extract of a luminous organ. The heating destroys the luciferase but does not harm the luciferin. Luciferase solution is prepared by making a cold water extract of a luminous organ, when both luciferin and luciferase dissolve and luminescence occurs. The extract is then allowed to stand in the air till the light disappears, evidence

that the luciferin has been completely oxidized, leaving the luciferase, an enzyme, in solution. Both solutions alone are dark, but when luciferin and luciferase solution are mixed, luminescence occurs. A luciferase solution, by virtue of its mode of preparation, must contain oxyluciferin as well as luciferase.

It is with such extracts as these that the ordinary chemical procedures of precipitation and re-solution have given us our knowledge concerning the properties of luciferin and luciferase. The most favorable animal for experimentation is a small ostracod crustacean, *Cypridina,* and subsequent remarks refer to material obtained from this organism.

In any experiments on the chemical nature of luciferin and luciferase two points must be kept in mind: (1) In the case of luciferin, spontaneous oxidation may occur without luminescence. Chemical manipulation must be very rapid or be carried out under conditions to prevent the oxidation of luciferin. (2) In the case both of luciferin and luciferase, very minute amounts of these substances will give a visible luminescence. Thus, if we assume 1 per cent luciferin in dried *Cypridina,* it is possible to show that one part of luciferin in 40 billion (40×10^9) parts sea water will give a visible light when mixed with luciferase. Making the same assumption of 1 per cent luciferase in the dried animal, one part luciferase in 8 billion parts sea water will give a visible luminescence when mixed with luciferin. The luminescence test is therefore a very delicate one.

Glassware used to hold luciferin solutions becomes contaminated with an adsorbed film of luciferin which seeps off in water and may introduce serious error in any exact quantitative determination.

The adsorption of luciferin by glass is shared by many finely divided substances such as bone-black, kaolin, talc, $CaCO_3$ and $Fe(OH)_3$. It is not so readily removed from solution by organic particles like those of caseinogen or corn starch.

Luciferase is also readily adsorbed by bone-black, $Fe(OH)_3$, As_2S_3, infusorial earth, talc and kaolin: much less readily by ground glass, sulfur powder, gelatin or agar-agar threads, heat-coagulated egg albumen, freshly precipitated caseinogen, cotton, and corn starch. Comparative adsorbability cannot be given, because, with different materials, uniform surface area is difficult to obtain.

Luciferin passes collodion or parchment membranes, but relatively slowly compared with salts like NaCl; luciferase does not pass either collodion or heavy parchment. After twenty-four hours dialysis, a slight amount of luciferase may pass parchment membranes; but when one considers the minute concentration of luciferase detectable, the amount which passes the parchment is negligible.

Both luciferin and luciferase pass porous porcelain, siliceous earth and alundum filter tubes.

Luciferase is insoluble in all except aqueous solvents. It is soluble in pure water and dilute salt solutions and in dilute acid and alkali solutions, although both acid and alkali injure it.

Luciferin is soluble in all aqueous solvents, pure water and dilute salt solutions, dilute acid and alkali. It is unharmed by dilute acid, but oxidizes readily in dilute alkali so that no more luminescence will appear upon adding luciferase. It is soluble in methyl, ethyl, propyl, and benzyl alcohols, acetone, .ethyl acetate, glycerol, and glycol; slightly soluble in isobutyl and amyl alcohol,

ethyl propionate, butyrate, valerate and nitrate; insoluble in ether, chloroform, carbon disulfide, carbon tetrachloride, benzene, toluene, xylene and petroleum ether.

Kanda has made use of the solubility in methyl alcohol to prepare a purified luciferin solution which gave no protein color reactions. If the procedure involved has not caused too great a diminution in the amount of luciferin, we have fairly good proof of the non-protein nature of luciferin. It must always be born in mind, however, that luminescence is a much more delicate test for luciferin than color reactions are for proteins.

Luciferin is not injured by any of the common enzymes such as malt diastase, ptyalin, amylopsin, yeast invertase, pepsin-HCl, trypsin, erepsin, rennin, steapsin and urease. The enzyme solutions were tested for activity on their normal substrate as controls, and also kept with luciferin, under anaerobic conditions, at 38° C. for 18 to 48 hours, using toluol as preservative to prevent bacterial growth.

Under the same condition luciferase is destroyed only by trypsin and erepsin. Pepsin-HCl could not be tested as the HCl alone destroys luciferase.

The significant result is the destruction of luciferase by the proteolytic ferment, trypsin, and the *stability* of luciferin under the influence of both trypsin and pepsin. This is additional evidence in favor of the nonprotein nature of luciferin, although some peptones are not attacked by trypsin. Luciferin might be such a peptone if argument is based upon this evidence alone.

Colloids are quite generally salted out of solution by saturation with alkali or alkaline earth metal salts. Luciferin and luciferase may also be salted out by appropriate salts. Table I shows the effect of adding salts to luciferin and luciferase solutions prepared by extracting *Cypridina* with water.

Kanda has obtained slightly different results in salting out the filtrate from a luciferase solution first precipitated with $HgCl_2$ in excess (see Table I). He finds "slight precipitation" on half saturation with $MgSO_4$; "complete precipitation" on saturation with $MgSO_4$ and also on one-half saturation with $(NH_4)_2SO_4$.

The "slight precipitate" is no doubt a matter of judgment, whether we are to call a turbidity a precipitate or not; the "complete precipitation" on saturation with $MgSO_4$, where I find nearly complete precipitation, may be merely a matter of dark adaptation of the eyes, or a question of saturation at different temperatures or acidities. But "complete precipitation" on half-saturation with $(NH_4)_2SO_4$, where I find "slight precipitation," requires some explanation.

I have repeated both Kanda's and my own procedures and find both our statements to be correct. With a luciferase extract in water there is only slight precipitation of luciferase; but with the filtrate from a mercuric chloride treated luciferase extract, half-saturation with $(NH_4)_2SO_4$ practically completely precipitates the luciferase.

The difference in result is possibly due to the fact that Kanda is precipitating the mercury salt of luciferase while I am precipitating luciferase itself.

Perhaps the best idea of the effect of heavy metal salts and the alkaloidal reagents can be gained by reference to Table I, which gives my own results on solutions of luciferase and luciferin obtained directly from the animal, and those of Kanda on luciferin and luciferase purified in various ways by preliminary precipitation and re-solution.

TABLE I. *Properties of Photogenic Substances of Cypridina Hilgendorfii.*

Property	Luciferase (H)*	Luciferase (K)*	Luciferin (H)*	Luciferin (K₁)*	Luciferin (K₂)*
SALTING OUT BY					
Saturation NaCl	Not precipitated	Not precipitated	Not precipitated	Not precipitated	Not precipitated
Saturation NaCl + acetic acid	" .... "		......	" "	" "
Half saturation MgSO₄	....	Slightly precipitated (?)	......	" "	" "
Saturation MgSO₄	Nearly completely precipitated	Completely precipitated	Partially precipitated	" "	" "
Saturation MgSO₄ + acetic acid	....	....	Partially precipitated	" "	" "
Half saturation (NH₄)₂SO₄	Slightly precipitated	Completely precipitated	Not precipitated	" "	" "
Saturation (NH₄)₂SO₄	Completely precipitated	Completely precipitated	Nearly completely precipitated	Completely precipitated	Completely precipitated
Saturation (NH₄)₂SO₄ + acetic acid	....	....	Nearly completely precipitated		
ALKALOIDAL REAGENTS					
Phosphotungstic acid	Completely precipitated	Completely precipitated	Very nearly completely precipitated	Completely precipitated	Not precipitated (?)
Phosphotungstic and acetic acids	....	....	Very nearly completely precipitated		
Phosphotungstic acid and HCl	....	....	Completely precipitated		Not precipitated (?)
Tannic acid	Nearly completely precipitated	(?)	Nearly completely precipitated	(?)	Not precipitated
Tannic and acetic acid	....	....	Nearly completely precipitated		
Tannic acid and HCl	....	(?)	Nearly completely precipitated	(?)	Not precipitated (?)
Picric acid	Nearly completely precipitated		Not precipitated	Not precipitated	

Substance	Luciferase (H)	Luciferase (K)	Luciferin (H)	Luciferin (K₁)	Luciferin (K₂)
Picric and acetic acids		Not precipitated	Not precipitated	Not precipitated	Not precipitated
Picric acid and HCl		″	″	″	″
$K_4Fe(CN)_6$		″	″	″	″
$K_4Fe(CN)_6$ and acetic acid	Completely precipitated	″	″	″	″
Phosphomolybdic acid		″	″	″	″
Phosphomolybdic acid and HCl		″	″	″	″
HCl		(?)			(?)
ACIDS AND ALKALIES					
NaOH	Not precipitated	Not precipitated	Not precipitated	Not precipitated	Not precipitated
NH₄OH	″	″	″	″	″
Acetic acid	″	″	″	″	″
H_2CO_3	″	″	″	″	″
Trichloroacetic acid		″	″	″	″
HCl, H_2SO_4 or HNO_3		″	″	″	″
HEAVY METAL SALTS					
Basic lead acetate	Completely precipitated	Not completely precipitated	(?)	(?)	(?)
Neutral lead acetate	Nearly completely precipitated	Not completely precipitated	Not precipitated	Not precipitated	Not precipitated
Neutral lead acetate and acetic acid		Not precipitated	″	″	″
Mercuric chloride	Not precipitated	Not completely precipitated	″	″	″
Mercuric chloride and acetic acid		Almost completely precipitated	″	″	″
Uranyl nitrate and acetic acid		Not completely precipitated	″	″	″
Copper sulfate			″	″	″
Zinc sulfate			″	″	″
Ferric chloride			″	″	″
Silver nitrate			(?)	(?)	(?)

* Luciferase (H) refers to a cold water solution of the entire animal containing luciferase and other substances. Luciferase (K) refers to a cold water solution of the entire animal precipitated with HgCl₂ to excess, and the filtrate used in the tests. Luciferin (H) refers to a hot water extract of the entire animal containing luciferin and other substances. Luciferin (K₁) refers to a methyl alcohol extract of dried fat-free *Cypridinæ* evaporated to dryness, and the residue dissolved in water saturated with hydrogen. Luciferin (K₂) refers to a methyl alcohol extract of dried fat-free *Cypridinæ* (containing luciferin) evaporated to dryness, the filtrate (containing luciferin) evaporated to dryness, dissolved in water, and salted out with (NH₄)₂SO₄. The precipitate of luciferin which forms is dissolved in water for the tests.

rôle of metals in vital reactions, has so intimate a bearing on the strictly physical properties of living matter, that its place in this chapter is justified. In the last analysis all properties and processes are physical.

The capacity to oxidize substances which under ordinary conditions undergo no such chemical change is one of the outstanding and distinguishing characteristics of living matter. It is the "fire" within us that keeps us alive. Every cell of a living thing contains such a fire. Commonly this combustion process is referred to as respiration. But oxidation phenomena in organisms are of two types, the irreversible, to which belong the energy-yielding processes of combusion, and the strictly reversible, such as the oxidation of the leucosubstances of echinochrome and hermidine [29]. The former class includes the chemical processes of respiration which seem to involve no true equilibria. The latter class has to do with true and reversible equilibria which are susceptible to measurement by the electrode method.

The initiation of precise quantitative methods of measuring oxidation-reduction processes is due to Clark [38]. He believes oxidation-reduction to involve a transfer of electrons from reductant to oxidant. Clark measured, in terms of electrode potential, the electron-fugacity, that is, the electron-escaping tendency, or reduction intensity. By a series of mathematical deductions Clark arrived at the formula:

$$E_h = E_o - \frac{RT}{nF} \ln \frac{(Red)}{(Ox)}$$

where E_h is the difference in volts between the oxidation-reduction electrode and a normal hydrogen electrode, E_o a constant characteristic of the system in question, R the gas constant, T the absolute temperature, F the Farad, n the number of electrons transferred in the reaction formula, ln the symbol for Naperian logarithm, and Red and Ox the concentrations of reductant and oxidant respectively.

By means of this formula it is possible to express relative oxidation-reduction intensities in terms of electrode potential. The potential of a system is, therefore, a numerical index of its oxidation-reduction intensity [159].

As a symbol for reduction intensity (as opposed to total amount of reductant present) Clark used rH to indicate an analogy with pH which refers to the intensity of acidity (as opposed to the total amount of acid present). The symbol rH indicates the negative logarithm of the hydrogen pressure in equilibrium with the oxidation-reduction system in question. By making a large number of electrometric observations on certain dyes Clark has been able to prepare a series of oxidation-reduction indicators, of which it is possible to say that their titration curves from complete oxidation (completely colored form) to complete reduction (completely colorless form) are related to definite pressures of hydrogen. With the object of finding the average rH of the cell-interior, Needham and Needham [159] injected, by means of micro-technique, various oxidation-reduction indicators into *Amoeba proteus*. By comparison with Clark's curves they found that the level of reduction corresponded to an electrode potential of $E_h = +0.097$, giving an rH value between 17 and 19. Since Clark had shown that pH effects the rH value, it was necessary, as a preliminary, to find the pH value for the cell by injecting suitable indicators. The value found was 7.6. A hydrogen-ion concentration (pH) of 7.6 and an oxidation-reduction potential (rH) of 17-19 are both near neutrality. The amoeba cell is, then, on the basis of the Needhams'

[160] measurements, slightly alkaline and slightly on the electronegative or reduction side of the oxidation-reduction neutrality.

Schmidtmann [197] and others have obtained somewhat lower values of protoplasmic pH (6.7 for muscle and liver tissue), indicating a more acid condition of the living cell. Needham and Needham [160] also found a more acid condition (pH = 6.6) in the eggs of echinoderms.

Warburg [230] and Meyerhof [146] observed enormous increases in the oxygen consumption of an egg at the time of fertilization. The Needhams [160] found that there is no change in internal pH at the time of fertilization of echinoderm and tunicate eggs; nor is there any change in rH on fertilization. These data are most striking in the face of the findings of Warburg and Meyerhof which reveal a 2000-fold increase in oxidation at fertilization. The data support Warburg's [229] earlier statement that rate of oxidation is independent of the oxygen pressure, i.e., of the oxygen concentration in the egg.

Brooks [21], by observing the penetration of oxidation-reduction indicators into the alga Valonia, estimated the reduction potential of the sap to be between 16 and 18. Brooks points out that the closeness of these values of the rH of plant cell sap to those of the protoplasm of amoeba suggests the possibility that all forms of life have approximately the same oxidation potential.

Irreversible oxidations, such as the complete combustion in the cell of sugar to carbon dioxide and water, cannot give steady electrode potentials and therefore cannot be expressed by the reversible electronic equations. Conant and Lutz [41] have shown that certain isolated oxidation-reduction processes may be characterized by the potential of the weakest reducing agent which will cause reduction to occur, or of the weakest oxidizing agent which will cause oxidation to occur. In the living cell it may be that a steady state of dynamic equilibrium is maintained between the oxidative effects of activated oxygen and the reductive effects which become especially evident under anaerobic conditions. If, in this dynamic equilibrium, there are included systems capable of having a decided effect on the electrode or on truly reversible indicators, the state of the equilibrium as a whole might be characterized by an electrode potential or by an rH value. Furthermore, it has been suggested by Cannan, Cohen and Clark [30] that conditions so characterized may determine the direction of specific reactions in the sense proposed by Conant and Lutz.

The rH of protoplasmic systems has been measured in several ways: 1, micro-injection of indicators [159, 186]; 2, electrometric measurements on cell suspension [30]; 3, vital staining with indicators [21]; and 4, electrometric measurements on thawing purée [227]. All the results obtained by these different methods are in close accord with each other and with the theoretical figure predicted by Wurmser [244]. Dixon [48] has recently indicated a manner of linking up the conceptions of Clark with those of Wieland on hydrogen transport.

Needham and Needham [161] have given a review of work on the oxidation-reduction potential of living tissues and cells.

Reference should be made to the extensive investigations of Crozier [43] on the use of the critical thermal increment (μ of Arrhenius) for the characterization of biological processes whose velocities are a function of temperature. In the case of oxidation phenomena critical increments of the orders 11,500 and 16,700 have been encountered. For the reduction of methylene

blue by bacteria through the removal of H from succinic acid, $\mu = 16,700$. It is suggested that iron very likely has a catalytic rôle in vital respiratory processes. The action of the hydroxyl ion is believed to be revealed in the value $\mu = 11,500$ which is frequently obtained in connection with respiration.

The part which iron plays in the mechanism of oxidation has been extensively studied by Warburg. Numerous researchers have given a prominent position to this metal as a factor in vital phenomena. Perhaps the speculations have gone too far, but coming as they do from investigators at work in quite varied fields, the possibility of iron having an important rôle, probably as catalyst, in living processes appears to be very likely. As for oxidation phenomena, it certainly seems to be true that no element plays a more profound part than does iron.

Warburg [232] believes respiration to be a cycle in which molecular oxygen reacts with bivalent iron, whereby iron in a higher state of oxidation is formed. The oxidized iron reacts with the living organic substance and is again reduced to bivalent iron. The amount of iron in different types of cells has been determined and found to be one tenth to one hundredth of a milligram per gram of cell substance. Warburg considers the possibility of other heavy metals functioning as the respiratory catalyst. Copper and manganese would apparently serve equally well. These elements occur in protoplasm but their amounts are too minute to account for the rate of oxygen consumption in respiration (for example, one gram of cell substance to one-ten-thousandth of a milligram of manganese, a hundred to a thousand times smaller than the iron content).

It is extraordinary to what extent heavy metals are found in organisms. Copper, ordinarily highly toxic to living things, is found in plants and animals in surprisingly great quantity. Mn, Zn, Ni, and Co are widely distributed in plants [144]. It is not unreasonable to assume that the rôle of these metals in life is a catalytic one.

The possible catalytic rôle of iron in photosynthesis forms a big chapter in the physiology of plants. The importance of iron for normal plant growth was first made known by Gris [86] who discovered the cause of chlorosis in plants. Molisch [151] more fully demonstrated the discovery of Gris. In the absence of iron, plants become chlorotic (lose their typical green color) because of their inability to synthesize chlorophyll.

That the primary synthesis of plant foods, of formaldehyde from carbon dioxide and water, involves iron functioning as a catalytic agent, is the opinion of Moore [152], Baly [4] and others. Baudisch [5] warns against regarding primary photosynthesis as a catalytic reaction and states that the process is a much more complex one than most workers appreciate.

The nature of the part which iron plays in vital phenomena is not fully known, but it does appear to have been conclusively demonstrated that the presence of this metal is a prerequisite to the proper functioning of many organisms.

The fact that the manganese content of normal green leaves may be from five to ten times that of chlorotic leaves, leads McHargue [144] to believe that manganese may replace iron as the essential element in the synthesis of chlorophyll. While we cannot deny to iron the important place which it holds in vital, especially respiratory, phenomena, yet the rôle of other elements—sulfur, for example, in glutathione processes—should not be underestimated.

One is led to conclude that in all organisms there exists a nicely balanced

association of elements, and that any omission, or other disturbance, which upsets this balance, will lead to pathological conditions.

Osmotic Pressure

Philosophical and experimental biology, no less than other fields of thought, have had their fads. The older generation of scientists have seen several dominating influences hold sway for a decade or more only to be supplanted by other lines of reasoning. Physical biology had its beginning with the work on osmosis by the botanist Pfeffer [180] whose experimental data were amplified by the deductions of Van't Hoff [226]. A few years later Traube [222] published his researches on surface tension, with the result that few vital processes which could be interpreted in the light of physical chemistry escaped without an attempt being made to explain them on the basis of surface tension. Adsorption next held the attention of biologists as an important factor in natural phenomena. It still plays a prominent part in biological thought. A fourth influence now holds sway, namely, that of electrical forces.

It should not be assumed that these theories, which have had a dominating but transitory effect on biological interpretations, have, in giving way to a successor, been necessarily discarded. On the contrary, each has firmly established itself as one of the physical factors which determine the behavior of organisms. The mistake of the enthusiasts at work at the time lay in their attempt to explain all but few physiological processes by the prevailing theory. So it was, in part, with osmosis, and even more so with surface tension, and some [64] are of the opinion that the present-day enthusiasm over bio-electric phenomena has likewise gone beyond the bounds of sound reasoning.

The rôle of osmosis (when considered apart from imbibition) in biological processes is of greater significance in the life of plants [127] than in that of animals. Many plant cells possess large central vacuoles. Turgidity in them is primarily the expression of the osmotic pressure of the sugar and salt solutions in the cell sap. This pressure maintains the turgor of plant tissues which, in turn, is to some extent responsible for the increase in size of young and still plastic cells, and therefore for growth.

Much has been ascribed to osmotic turgor for which it is not responsible. There are, nevertheless, many processes in plant life of which change in osmotic turgor is the cause, as is the case of that nicely adjusted mechanism which folds the leaves and lowers the petiole of the sensitive plant, *Mimosa pudica*. A contact stimulus at the tip of the leaf is transmitted, closing the leaflets as it goes, to the base of the petiole, where it is received by the pulvinus, a swelling in the axil of the leaf stem. Here is located the machinery for moving the petiole. Exosmosis of water from the cell vacuoles (and from the protoplasmic jelly itself) takes place, lowering turgor, thus causing contraction of the pulvinus tissue and falling of the petiole. The loss of water which brings about decreased osmotic turgor is due to increased permeability of the vacuolar (and the outer protoplasmic) membrane, for water. How this change in permeability is brought about, is not known. It is induced by the arrival of the stimulus from the tip of the leaf.

The turgor or plumpness of mature plant tissues is undoubtedly due to the osmotic pressure of the cell sap, a salt and organic solution surrounded by a differentially permeable membrane. But growth in plants is also the expression of the imbibition pressure of the living jelly itself. There are, of

course, many factors involved in growth, imbibition or osmotic pressure being but one of them.

IMBIBITION

Had these pages been written a dozen years ago, it could have been said that whatever the precise nature of swelling in jellies may be, the mechanism of it is quite distinct from that of osmosis. But today, while some still hold to the above statement, others are of the opinion that imbibition in jellies is, in part, an osmotic phenomenon.

The taking up of water by gels and jellies involves at least two fundamentally different types of mechanism. In non-turgescible gels, such as that of silicic acid, the absorption of water is probably a pure capillary phenomenon. The taking up of water by such gels is not rightly regarded as an example of imbibition, a term which carries with it the concept of swelling. The extraordinary amount of water (99.8%) in the non-turgescible cystine gel described by Gortner [81], or in a cadmium [214] or an iron-oxide [195] gel, is probably held in a purely mechanical way as capillary water. Yet in turgescible gels, capillarity can hardly be the primary factor involved, since it is produced by surface tension which is not a distending force. Nor can capillarity play a part in the further swelling of a jelly which is first swollen to maximum distention in water and then swells more when acid is added. There are no capillary spaces left to fill to account for the additional swelling. It is probably here that the Donnan [50] effect comes into play.

There is, however, some ground for regarding capillary forces as, in part, responsible for the taking in of water by turgescible gels. If jellies are entanglements of fibrous structural units, there is probably water immeshed between the fibrils. Such capillary water is mechanically held. Alexander [1] and others believe gelatin gels to contain "tiny pores." If this is true we must grant that capillarity is a force involved in the hydration of turgescible gels.

The modern viewpoint looks upon imbibition as, in part, a Donnan equilibrium effect, and therefore, in a sense, osmotic. The diffusible ions (in gelatin immersed in dilute HCl) are H and Cl; the non-diffusible ions are gelatin, and the "membrane" is the force of cohesion between the gelatin ions of the solid jelly.

There is some objection to terming such a process osmosis, since the latter has been clearly defined as the diffusion of a solvent through a morphological, differentially-permeable membrane. No such system really exists in a block of gelatin. But the measurable results indicate so close a similarity between the two processes, that it seems likely that the one, imbibition, involves, in part, the other, osmosis. The work of Procter [182], Wilson [241], Loeb [135], and Northrop [167] points to this conclusion.

Those who regard the swelling of gelatin as essentially an osmotic phenomenon, look upon the two processes as identical as far as the forces involved are concerned. The force which draws water into gelatin is the same which draws water into a sugar solution surrounded by a differentially permeable membrane.

Wilson [242] interprets swelling in the following way. Gelatin immersed in hydrochloric acid forms gelatin chloride, the gelatin cations of which form the network of the jelly and are not in solution in the same sense as are the chloride ions which remain in the interstices of the network. Since these

chloride ions are balanced only by the positive gelatin ions, there results an unequal distribution of all ions between the external solution and that absorbed by the gelatin such that the total concentration of ions is greater in the jelly than in the surrounding solution. In tending to diffuse into the external solution the anions of the protein salt exert a pull upon the cations forming the gelatin network causing an increase in volume of the jelly which is the imbibition pressure.

Northrop [167] thinks gelatin is a mixture of at least two substances, one of which is soluble in cold water, does not form a gel, and has a high osmotic pressure; the other is insoluble in cold water and forms a gel. The swelling pressure is due to the osmotic pressure of the soluble constituent of the gelatin held in the network of insoluble fibers. Salts increase the swelling of gelatin [166] just as do acids [135]. Northrop [166] believes this to be due to an increase in osmotic pressure which is the result of a change in the osmotic pressure of the (soluble constituent of) gelatin, rather than to a difference in ion concentration. The swelling of gelatin in water or in salt solution is, then, in the opinion of Northrop [167], an osmotic phenomenon, just as Procter and Wilson [182, 183] believes to be true in the case of acid swelling. Walter [233] in his studies on turgor in plants concludes that there is no difference in principle between swelling and osmosis; one process passes gradually over into the other.

Another possible interpretation of swelling is that involving those forces which are responsible for water of hydration, whether adsorption, or primary or secondary valence. The tendency of molecules and ions to surround themselves with water of hydration is common. Mobility of ions is determined by the thickness of the water shell which each ion drags with it and which varies in size with different ions, as their migration numbers show.

The manner in which water is held in a protein jelly may be essentially the same as that involved in hydrated crystals. The older and still prevalent idea of the nature of water of crystallization is that the bond between water and crystal molecule is one of secondary valence or adsorption, that is, that the water molecules attach themselves to other molecules in the solid and are readily separable, for example, by heat. The newer viewpoint sees no special clear identity of the water molecule. The crystal with the water is a new compound altogether, with a new arrangement of the atoms, which now includes certain multiples of $2H + O$. It does not follow that because H_2O is given off as such, water is existent as a molecule in the crystal.

Other possible explanations of imbibition exist. The water may be held by electrical forces existing at the surface of the protein particles. Evidence tending to prove this is to be had from the extremely rapid change in viscosity which takes place in a jelly when minute quantities of electrolytes are added. The change is so rapid that diffusion ("osmosis") could hardly play a part.

Imbibition pressure may be osmotic, or capillary, or the expression of adsorption or electrical forces, or a combination of several of these.

Young embryonic plant tissues, in which growth is most active, are not highly vacuolate; consequently, growth in plants must to a great extent be due to imbibition pressure. This is to be expected when one realizes the tremendously high imbibition pressures of certain proteins and carbohydrates.

The mechanical force involved in distention, that is, in growth, in animals, is imbibition pressure. A jelly-fish attains adult size through increase in water content, to a maximum of over 99 per cent, by means of imbibition. Imbibition

pressure in animals does not appear ever to reach very high values, as the flaccidness of animal tissues attests.   .

The distinction here made between the rôles of osmotic turgor and imbibition pressure in living systems, does not even tacitly deny the possible identity of imbibition and osmosis in jellies, but it does emphasize the evident distinction between the two types of systems which produce distention in organisms; the one an osmotic system comprising a salt and organic solution surrounded by a differentially permeable membrane, and the other, an elastic jelly, the protoplasm itself.

Imbibition is quite independent of osmotic pressure as regards the respective physiological rôles of the two. Thus, seeds will, by imbibition pressure withdraw water from saturated lithium chloride (osmotic pressure 1000 atm.) although their salt content is sufficient to account for only a few atmospheres of osmotic pressure [80]. But osmotic pressure in plants .may reach very high values. Xerophytes of salt marshes attain a maximum osmotic value of 172 atmospheres. But still greater are possible maxima of imbibition pressure. Starch will swell under a pressure of 2523 atmospheres.

An interesting and important economic problem is that of winter hardiness of organisms, which apparently has to do with the percentage of bound water of imbibition.

Water in protoplasm is undoubtedly in two states, bound and free, the former comparable to the water of crystallization of hydrated crystals and to the firmly held "adsorbed" water of imbibition of jellies. There is a marked difference in the behavior of these two states which is of fundamental importance to life. It appears that the resistance of seeds and insects to the cold of winter is a function of the percentage of bound water. Newton and Gortner [164] have shown that winter hardiness of wheat is directly correlated with amount of bound water. Robinson [189] has established a similar relation for insects which gain in bound water and lose in free water with falling temperature. He further points out that bound water will not conduct electricity, will not dissolve sugars, and will not freeze at five degrees below zero F.

It is evident from the above that the greater the quantity of free water which can be converted into bound water and the more rapidly this can be done, the greater is the resistance of that species to the winter's cold.

Some interesting work is being done by Lucké and McCutcheon [137a] on the irreversible and reversible swelling of protoplasm. The first type is occasioned by such agents as hydrochloric acid, ether, and heat, and results in death. The second type of swelling is caused by hypotonic sea water and is not only reversible but the unfertilized sea urchin eggs when returned to ordinary sea water regain their normal size and are capable of fertilization and subsequent development.   .

SURFACE TENSION

The great fount for explanations of natural phenomena which can be interpreted on a physical basis, has, during the past two or three decades, been surface tension. To Traube [222] do we owe the initiation of the surface tension impetus which went so far in biological reasoning. Amoeboid movement, muscular attraction, nerve impulses, cell division, protoplasmic streaming, gastrulation, permeability changes, etc., etc., have been, at one time or another, regarded as surface tension phenomena. That surface tension may

play a part in some of these processes cannot be denied. It would be quite extraordinary if it did not. But that it is the ultimate or chief factor involved in all cases where it has been applied is most certainly untrue. As a matter of fact, very few instances indeed, among all those in which surface tension has served as the physical explanation of a process, remain which cannot be equally well or better interpreted on the basis of some other mechanism.

As regards gastrulation, while it in all probability involves surface tension changes, these would appear to be effects and not causes. The most suggestive speculation on the mechanism of the phenomenon is one based on the work of Hatschek [90] on change in form of elastic gel bodies with drying. Hollow sphere segments of gelatin "gastrulate" on drying, just as do living embryos. Dehydration, then, rather than surface tension, is possibly the mechanism involved in the invagination of the gastrula.

It has just been stated that it would be quite extraordinary if surface tension were not a factor in certain vital processes, as for example, in the formation of pseudopods of amoebae and myxomycetes. We have reference here to ordinary mechanical surface tension. If the tension at the interface between protoplasm and water is uniform over a more or less spherical mass of protoplasm, and if this tension is reduced at any one point, the expected result would be, if there exists an internal osmotic or imbibition pressure, the formation of a pseudopodium, or of any similar protoplasmic extension at the surface. But if the formation of surface protrusions on protoplasm were a pure surface tension procedure the extension formed should be globular, or at least approach the shape of minimum surface. This is seldom the case. The fundamental law of minimum surface is not obeyed by protoplasm. The surface of protoplasts is of all sorts of contours, and protoplasmic protrusions assume a variety of fantastic shapes. The substance is alive, dynamic, and not passively amenable to ordinary surface tension laws.

Gibbs' principle of surface adsorption is frequently cited by biologists in an interpretation of vital processes. Yet how far are we justified in assuming that this law of Gibbs' is fully applicable to the living system? It was formulated in reference to the behavior of true solutions. Does it therefore hold for colloidal jellies with a semi-rigid structure? If so, then certainly in some modified form.

It should not be assumed from the foregoing that the protoplasmic surface is exceptional in lacking surface tension. The implication is only that diffusion and surface tension laws as founded on true solutions do not fully explain the behavior of colloidal jellies.

The problem of how far the laws of physical chemistry can be applied to protoplasm, to a system which includes infinitely more variables than the system for which the laws were stated, is one of the most important problems with which the biologist has to deal today.

That certain fundamental physical laws do not alone explain the behavior of protoplasm, is well illustrated in the law of diffusion, that the rate of diffusion of a solute in a solvent is inversely proportional to the size of the molecule of the solute. In protoplasm this is not always the case. Salts often enter the cell more slowly than do large organic molecules. Adsorption phenomena apparently come into play [46]. The law of diffusion was formulated to interpret the behavior of true solutions, and does not, therefore, necessarily tell us anything about colloidal jellies.

While surface tension is not usually the sole or primary force involved

in vital processes, as has often been assumed, yet it is a force to be considered. Du Noüy [169] has made many interesting determinations of surface tension changes in sera and other colloidal solutions. He has found a spontaneous and rapid decrease of the surface tension of a serum in functions of the time, which may be expressed by the exponential formula: $\gamma = \gamma_0 e^{-Kt}$ where γ is the surface tension at the time t, γ_0 the surface tension at the beginning of the experiment, and K a constant.

Membranes of various thicknesses, from delicate imperceptible (when living) surface layers [105], to more substantial pellicles [204] are formed on the surface of protoplasm. It seems very probable that their origin on the living substance is due in part to the action of surface tension; but protoplasm is an infinitely more complex medium than is a pure solvent-solute solution. Membrane formation must, therefore, involve other forces than surface tension.

Many natural processes in living systems have been interpreted as surface tension phenomena. It would be of little value to discuss the numerous theories. Some, such as the explanation of protoplasmic streaming on the basis of surface tension changes, are the product of vivid imaginations.

The crawling movement, so called amoeboid movement, of "naked" masses of protoplasm such as amoebae and myxomycetes, is one of the outstanding cases in biology where surface tension has served as a basis of interpretation of the mechanism of the phenomenon. Much has been written about it [188] but the evidence in favor of it is weak.

Jennings [108] came to the conclusion that locomotion of amoeba is demonstrably not due to a local decrease in surface tension on the side toward which the animal is moving. Rand and Hsu [185] believe the observations of Rhumbler [188] on direction of currents in amoeba to be correct. It was these observations of Rhumbler which led him to support a surface tension hypothesis of amoeboid movement.

Another classical case in which surface tension has been resorted to as an explanation, is that of cell division, both direct (fission) and indirect (mitosis). The simple method of cell division, by pinching in two, certainly appears to be a surface tension process. It may be, but the proof is lacking. Far more complex is mitotic cell division, one of the most baffling problems in biology. That surface tension may be a process involved has been shown by Němec [162]. Other physical phenomena have been resorted to in an attempt to explain mitosis, such as magnetism, and solation-gelation—all interesting and instructive, but highly speculative.

The extent to which surface tension has been invoked in the interpretation of natural phenomena is well illustrated in the full discussion of the subject by Macallum [139] who made use of the wide presence of potassium in plant and animal tissues as a means of determining the occurrence of adsorption and, therefore, of differences in surface tension. Macallum believes the Gibbs-Thomson principle of surface condensation is fully applicable to living matter and serves in helping to explain the causation of certain vital processes.

A Dublin physicist, Fitz Gerald [66], was apparently the first to suggest that muscle contraction is a surface tension phenomenon. Since then others have advanced the same hypothesis, without, however, being in agreement on whether the contraction involves a decrease or an increase in surface tension. On the basis of the distribution of potassium and haloid chlorine

In muscle fiber, Macallum [139] concludes that the simplest explanation of the shortening of the sarcous element is that surface tension on its lateral wall decreases. He adds, that alteration in shape of the doubly refractive disc makes it imperative to believe that surface tension is concerned, and that redistribution of potassium can be explained in no other way than through the alteration of surface tension.

Macallum is further of the opinion that intestinal absorption, and the excretory function of the kidney are essentially surface tension phenomena. He believes that in tissue absorption, secretion, and excretion, the Gibbs-Thomson principle continuously operates.

The culmination of Macallum's surface tension theories comes in his explanation of nerve impulses. He says the nerve impulse is always accompanied by, if not constituted of, a change in electrical potential which is initiated by progressive distribution of potassium ions along the course of the axon, and this change in potential would occur as often as a change of tension could take place on the surface of the nerve cell. On the basis of this hypothesis anaesthesia and narcotism are to be interpreted as involving a reduction in surface tension of all cells affected, but especially of nerve cells. Macallum believes that thermodynamic and chemodynamic processes and intrinsic pressure play a part, but are subordinate to the force of surface tension in psychic functions. He concludes that sensation is fundamentally and primarily a result of alteration of surface tension in nerve cells, and that memory itself may arise from adjustments in surface tension of cells in centers of the cerebral cortex.

One can only express wonder at the wide use of surface tension in the physico-biological reasoning and add the hope that some few of the speculations may prove to be true.

ADSORPTION

Adsorption has played a prominent part in theories of natural phenomena. In the main, these theories have withstood the test of time and opposition better than some others. The most severe criticism which adsorption as a factor in living and non-living processes has received, came from Loeb [135] who all but denied the very existence of the phenomenon—he avoided the word whenever possible. It may be that the behavior of certain jellies does not rest on adsorption properties, that there are no such structures as micellae in firm hydrated gelatin, and that if such units are present chemical action is not restricted to their surface. Thus would adsorption play no part in the reaction between gelatin and electrolytes. It may also be true that Freundlich's [70] adsorption isotherm does not hold for all proteins, and that the chemical reactions of some proteins are stoichiometrical and follow the laws of classical chemistry. Langmuir [116] has shown that the Freundlich adsorption formula does not hold for the reaction of mica, glass, and platinum; the forces here are purely chemical ones of primary and secondary valency.

Recent research tends to show that some work, which has led to a rejection of some fundamental colloid-chemical principles in favor of the theory of electrolytic dissociation, must be reconsidered. Kruyt and Tendeloo [115] have shown that the iso-electric point of gelatin does not depend on a definite hydrogen ion concentration (pH = 4.7) as Loeb [135] thought, but that this point can be reached at various hydrogen ion concentrations by adding

a quantity of another electrolyte ($K_3Fe(CN)_6$) just sufficient to produce discharge.

The work of Gortner and Hoffman [82] on wheat proteins lead them to the conclusion that the binding of protein with acid and alkali is both a stoichiometrical chemical and a colloidal adsorption one, depending on H-ion concentration. The chemical type of combination takes place between pH 2.5 and 10.5, and the amount of acid or alkali bound depends on the chemical composition of the protein. The adsorption type of combination takes place below pH 2.5 and above pH 10.5, and all proteins at these pH values, regardless of their chemical composition, bind approximately the same amount of acid or alkali.

Perhaps a definition, or an example which is more easily given, of adsorption is advisable before an attempt to prove its presence as a factor in biological processes is undertaken. The removal of a dye, such as Congo red, from its aqueous solution by filter paper is a simple case of what is commonly referred to as adsorption. The ease with which the dye is then removed from the paper, and the unlikelihood of any chemical reaction taking place between the dye and the cellulose, is evidence of simple physical adsorption. Another classical example of adsorption is the reduction in concentration of an acetone solution when shaken with charcoal. The acetone is adsorbed to the surface of the charcoal particles. We might also refer to the extensive use of charcoal, and now of silica gels [175], for removing vapors from air, purifying liquids, etc. We cannot, of course, always be sure that a chemical union of some sort has not taken place. Thus, the adsorption of congo red by filter paper depends on the calcium content of the paper and on the formation of a calcium compound of congo red [147].

There are undoubtedly all degrees of firmness of bond between very loosely adsorbed substances and those whose union has resulted in the formation of a new chemical compound. We can best accept Michaelis' [148] interpretation of adsorption as indicating those phenomena in which substances accumulate at the boundry layer, no implication being made as to whether the bond holding them there is physical or chemical, if indeed such a distinction can in any case be clearly made.

The problem is not a simple one. Further discussion would involve a consideration of the distinction between chemical reactions of primary and secondary valencies and of adsorption, which would cover many pages and lead to no final statement.

Sound reasoning points to the conclusion that adsorption is of paramount importance in vital processes. It is quite possible to consider the dynamics of physiological processes which involve adsorption even though the precise nature of what we call adsorption is not known. We shall content ourselves with a brief statement of some natural phenomena of which adsorption is apparently the mechanism.

Of fundamental and far-reaching significance in medical physiology and biological physics is the theory of respiration developed by Warburg [231, 232]. Following up some experiments of Freundlich [73] on the adsorption of oxalic acid by blood charcoal, Warburg, in searching for a possible chemical explanation, found that oxidation of the oxalic acid into carbon dioxide and water takes place. He then established that this oxidation can be retarded by narcotics just as one can retard cell respiration. Further, the retardation of these inanimate oxidation processes by narcotics rises with

the adsorption constants of the narcotics used (methyl-, ethyl-, propyl-, and phenylurethane). Warburg regards the retardation of oxidation as a consequent of a diminution of the free adsorptive surface of the carbon, and of the particles in the blood corpuscles or in any living respiring cell, due to adsorption of the narcotic.

Narcosis, then, if Warburg's ingenious theory is correct, is an adsorption phenomenon. The same is probably true of the extraordinarily high toxic effect of minute quantities of copper salts and aniline dyes, which led Nägeli [158] to regard the effect as of a special oligodynamic nature.

Czapek [46] believes "diffusion" of salts in the protoplast is not a process of diffusion but of adsorption. Ions of the high molecular salts are taken up by the cell more rapidly. What is probably meant is that diffusion of all ions takes place (otherwise no ions would enter) but that the high molecular salts are selectively adsorbed.

The question of the extent to which the physical properties of true solutions, such as diffusion and surface tension, are characteristic of jellies can be emphasized again here. Liquid jellies possess a structure in virtue of which they have such solid properties as elasticity and rigidity. How far are solution properties, such as diffusion, capable of manifesting themselves in systems possessed of a semi-rigid structure? The degree of elasticity (the degree of fixity of structure) in jellies, and the time, are factors which in part determine to what extent solutions laws are applicable to living and nonliving jellies.

Diffusion of ions, of molecules, and of aggregates of molecules must undoubtedly take place in protoplasm. This conclusion one cannot escape; but that diffusion is wholly free and unrestricted is quite improbable. The differentially permeable properties of the protoplasmic membrane is but one instance in support of the restricted and selective diffusion of ions in living cells, which is apparently, in part at least, determined by adsorption phenomena.

Herzog [99] and Freundlich [71] call attention to an interesting case of the very gradual but complete chemical change in a tissue by adsorption. The chitin plate of crustacean chelae is developed from sinew. The plate, although in construction quite different from sinew, has throughout the structure of the sinew from which it was evolved. Similar changes, the first step in which are adsorption processes, are found in the non-living organic and inorganic world. Zeolite undergoes such a change, and the transformation of cellulose into nitrocellulose is another instance. Freundlich [71] has given the name "permutoid" to these substances, and emphasizes the possibility of clearly distinguishing true chemical reactions from adsorption phenomena in the permutoids.

The investigations of Harvey [91] on luminescence in organisms (in the crustacean *Cypridina*) indicate that the process is one in which oxidation of a solution of luciferin is accomplished through adsorption by the particles of a colloidal dispersion of luciferase. (See Harvey's paper in this volume.)

The importance of adsorption in colloid-therapy is interestingly discussed by Alexander [3].

ELECTRICAL PHENOMENA

Electrical phenomena in living tissues are of two more or less distinct kinds. The first includes electromotive forces. These give rise to electric currents in nerve tissues, to membrane potentials, and the like. The second

involves what Freundlich has grouped under the term "electrokinetic" phenomena, and includes electrophoresis, agglutination, and kindred processes.

Electromotive forces. The idea that life and electricity are intimately associated has long been held. This belief is ridiculed by some [64], but others [68] are convinced that certain vital phenomena are electrical in nature. The discovery by Galvani that a frog's muscle contracts when brought into contact with the free ends of joined strips of copper and zinc was the first attempt to experimentally prove the electrical nature of life processes.

The experiment of Galvani did not demonstrate that muscular contraction is electrical in nature, but that contraction can be produced by electrical stimulation. The current is not, as Galvani thought, resident in the muscle. The salts and water in the muscle serve as an electrolytic solution which, with the two metals, produce a typical Volta cell. It remained for du Bois-Reymond to prove that organic tissues generate electric currents.

The experiments of du Bois-Reymond [16] laid the foundation of electrophysiology. He found that there is a flow of current in plant and animal tissues which are wounded; that the direction of this injury current is from the wounded to the normal region, and that the electromotive force is of the order of 40 millivolts.

Du Bois-Reymond's explanation of the origin of electric currents in tissue was his so-called "molecular theory" which presupposed the presence of tiny particles in protoplasm which are negatively charged on one side and positively on the other. Normally the particles are in electric equilibrium. A wound causes disturbances in their symmetrical arrangement and a current results. As fantastic as this speculation may seem, it is not far removed from the present day conception of charges on colloidal particles [225], the electrophoretic movement of which brings about differences in potential in living and non-living systems.

The theory of the origin of electrical currents in living tissues at present accepted, explains the phenomenon on the basis of the difference in potential which is known to exist on the surface of a membrane separating two salt solutions. The theory in its original form was first advanced by Ostwald [172] who believed that precipitation membranes are selectively permeable to ions. If the cation can readily pass through the membrane and the anion not, a potential difference would naturally be established between the two sides of the membrane, since the latter separates electrolytic solutions not in equilibrium. It is believed that the protoplasmic membrane is selectively permeable to ions. The theory of Ostwald was an important step toward putting physiology on a physical-chemical basis. The theory in its original form has not been experimentally verified, but has led to modifications by Nernst [163], Haber [87], Beutner [9] and others.

On the basis of the Nernst and Haber proofs that there exists a phase-boundary potential at the interface between electrolytic solutions, that is, that membranes separating salt solutions are the seat of potential differences, we must admit the strong possibility that electrical forces play a part in physiological processes, since in a heterophasic system such as protoplasm the number of phase boundaries, of membranes, protein or fatty in nature, is innumerable, and each, on the Nernst-Haber hypothesis, is the seat of an electromotive force.

The use to which the presence of electrical forces in tissues has been put as a source of explanation of vital phenomena, has not been as great as one

might imagine. The difficulty lies in proving one's speculations. An outstanding case and one which has been rather widely accepted, is the theory of protoplasmic (nerve and muscle) transmission advanced by Lillie [126] and others. The hypothesis of Lillie is drawn on a similarity between protoplasmic transmission and the transmission of activation in passive metals. (See paper by R. S. Lillie, this volume.)

Lillie [125] believes that in both cases the effect is dependent upon the properties of the membrane formed at the interface between the protoplasm, or the metal, and the surrounding electrolytic solution.

Ostwald [172] likewise saw in the electrical properties of precipitation membranes a possible explanation of electrophysiological phenomena. He said, "We may here express the opinion that not only muscle and nerve currents, but also the mysterious action of electric fishes may be explained by the properties of semipermeable membranes which have been referred to."

Certain physiologists regard the nerve impulse as an electric current traveling along the fiber in the same manner as such a current is conducted along a metal wire, depending on the differentially permeable state of the membrane surrounding the fiber or wire, and on the potential resulting therefrom. Others [68] recognize only a similarity in the two cases.

Ettisch and Péterfi [54, 55] have attempted to establish and measure differences in potential within a single cell. By means of a Zeiss-Péterfi [178] micromanipulator and with specially constructed microelectrodes, they sought for potential differences in amoeba. The results were negative. They state that in *Amoeba terricola* it was not possible to find two points which had between them a direct measurable potential difference, i.e., there is in this organism, at rest, no ionic equilibrium.

Taylor [220] has made successful determinations of the difference in potential between the interior and exterior of organisms by means of microelectrodes. He finds a potential difference of 0.002 volt between the cell sap in the vacuole of the green alga *Valonia* and the surrounding sea-water, the charge of the cell sap being plus with reference to the sea-water. The protoplasm of the ova of the echinoderm *Clypeaster* has a potential difference of 0.001 volts, and is minus with respect to sea-water.

Brooks [25] and Gelfan [79] have made measurements of the electrical conductivity of protoplasm. The former found that the plasmodium of the slime-mould *Brefeldia* has a resistance of 19,000 ohms, the equivalent of 0.00145 N NaCl. Gelfan found the cell sap of *Nitella* to have a specific conductance in reciprocal ohms of 0.0077, which is the equivalent of 0.07 N KCl, and the protoplasm of *Paramecium* to have a specific conductance of 0.0068. The average of Gelfan's measurements place the specific conductance of protoplasm at about 0.005 w^{-1}, while Brooks' findings translated to the same terms indicate a value of 0.0005 w^{-1}.

The electrodes used in the above experiments are of fine platinum in quartz and of agar in capillaries; the latter believed to be non-polarizable.

The effect of an electric current on tissues has been extensively studied. That there should be some pathological change is evident from human experience. Bersa [7] finds that the growth of roots is reversibly retarded by an electric current. He believes that this inhibition is due to permeability changes. Dixon and Bennett-Clark [49] likewise find that electric currents cause change in permeability. Cattell [31] has electrically activated Nereis eggs, causing them to develop into early larval stages without fertilization.

Interesting in connection with the rôle of electric currents in vital processes are the attempts in Europe and America to stimulate plant growth by static electricity of high voltage. While the European investigators [131, 109] make big claims for increase in crop yield, careful experimentation covering a period of years under different conditions in America, gives no indication that plant growth or crop yield is at all influenced by static electricity of 50,000 volts [18].

Membrane Potentials. That the origin of electric currents in living matter is believed to be the potential existing at the surface of membranes has already been stated. Ostwald [172] was the first to suggest this and to point out that membrane potentials in general are probably due to selective permeability of the membrane for ions. This theory is still adhered to in the main by some [149].

Classical experimental material to demonstrate membrane potentials is the apple skin [9]. A calomel electrode is connected (by means of an agar bridge) with a salt (0.1M KCl) filled cavity in an apple, and another electrode is similarly connected with a salt solution (0.01M KCl) in which the apple rests. The electrodes are joined to a cell, a potentiometer, and a galvanometer. On establishing electrical connection, a current of some 40 millivolts is detected. Similar membrane potentials are gotten when other tissues (frog skin, leaves, etc.) separate different concentrations of electrolytes.

The physical mechanism of the potential differences established is the only phase of this large and important subject which will be considered here.

A membrane which is the seat of electromotive forces can be regarded either as involving merely two surfaces—the potential produced existing at these surfaces—or as an intimate part of the solutions which it separates, being fully permeated by them. From this latter viewpoint the potential difference exists *within* the membrane, there being a gradual rise or fall in potential in passing through the membrane from one surface to the other. It is the second of these two hypotheses that Michaelis and Perlzweig [149] adopt. They regard the membrane as an enormously enlarged surface of the solution.

The difficulty involved in the first of the above two points of view is that of visualizing the physical mechanism of the passage of one kind of ion through the membrane; and it is on the assumption that the membrane is permeable only to cation (apple skin) or anion (red blood cell) that certain hypotheses of membrane potential are built.

The problem of getting only one kind of ion through membranes is an important feature not only of membrane potential theories, but of permeability theories as well. These two phenomena will probably be found to involve one common mechanism.

Authors of membrane potential and permeability speculations, who postulate the more ready passage of cation or anion, have not always made it clear how only the one kind of ion can get through the membrane. It is not possible to separate an ion from its mate by so great a distance as the thickness of a precipitation or protoplasmic membrane. In the blood corpuscle the pellicle is 0.5-0.8 μ thick [120, 204]. The separation of ionic couplets is a plausible interpretation of potential differences existing in solutions, yet when the interionic distances greatly exceed atomic dimensions there are certain to be set up powerful and disturbing electrostatic forces, which for distances equaling those of morphological membranes (0.1 to 1.0 μ) would reach thou-

sands of volts. It has been estimated that such forces would equal ten million volts if the interionic space were 1 cm. in a normal electrolytic solution.

The value of K in the Debye-Hückel [47] equation, which is the symbol for the radius of the ionic atmosphere in an electrolytic solution, gives an indication of the order of magnitude of interionic space. In a normal solution the value of K is 10^{-8} cm. (1Å). This is of atomic dimensions. (The limit of microscopic visibility and therefore of observable protoplasmic membranes, is about 0.1 μ or 100 Å.) So far as maintaining a balance is concerned, there is no difficulty. An interchange of ions will keep the system in ionic equilibrium; but the problem involves more than this, the getting of one ion through a membrane which measures not much less than one-half a micron. It can be accomplished only by giving the ion a new mate, or by assuming that that part of the protoplasmic surface which determines permeability is of atomic dimensions, which is the belief of Fricke [77]. It seems very unlikely that the extraordinarily complex mechanism of permeability control should lie in a monomolecular layer. The assumption is precluded if we grant that the first barrier to ions is the external oil film on protoplasts.

An electrostatic hypothesis of membrane potentials and permeability has been advanced by Raber [184] and Michaelis [147]. As an hypothesis of the physics of membrane potentials, the conception of the membrane as an electrostatic sieve serves well. As an explanation of protoplasmic permeability, the hypothesis is sound within limits, but it is insufficient.

That the apple skin is more freely permeable to cations than anions is the accepted belief, but permeability is possible only when a balance can be maintained by interchange. Michaelis and Fujita [148] found that not enough potassium leaves an apple in distilled water in two weeks to be detected, but if the apple is put in a solution of any sodium salt there is an outward diffusion of potassium, made possible because then the potassium can change places with another cation.

The apple skin appears to be impermeable to anions. This selective permeability is explained on the hypothesis that the membrane pores are positively charged and therefore hold back the anions by adsorption, while the cations, being of the same charge as the membrane, are repelled and are therefore free to pass through, from a region of greater concentration to one of lower concentration. As a result, the more concentrated solution is left with a negative charge and the less concentrated becomes positive.

We have solved the difficulty of getting one kind of ion through a membrane by, in a sense, doing away with the membrane, that is, while there can be no doubt of the presence of morphological membranes on protoplasm, these membranes are intimate parts of the living system as a whole. The diffusion potential postulated by Michaelis and Perlzweig [149] is possible because they regard the collodion membrane as forming a gradual transition between the two solutions. In the same way should the protoplasmic membrane be regarded. To be sure, the protoplasmic membrane is not as simple a structure as originally thought; we must apparently consider an outer fatty layer, an intermediate pellicle, and an inner surface, in at least some cases [105]. How much of this is living and how much secretion products, cannot be said; but it is clear (from observations to be given later) that the protoplasmic membrane collectively is capable of the same physical changes (in viscosity, etc.) as is the inner protoplasm, and is, therefore, to be regarded as much a living thing as any region of the protoplast, of which it is a part.

Ions penetrate the protoplasmic surface by the same mechanism that they diffuse through the protoplasmic mass.

Electrophoresis. One of the most active lines of research in colloidal physics in recent years has had for its object the determination of the magnitude and nature of the charge on colloidal particles. Much of this experimental work has dealt with electrophoretic phenomena (cataphoresis). Its influence on biological studies is just beginning to be evident, but while electrophoretic investigations on living systems are still in their infancy, there is already an indication that they will lead to far reaching theoretical conclusions, and to findings of great value to medicine.

Experimental work points to the conclusion that most living cells and microscopic organisms (blood corpuscles, bacteria, unicellular algae) are negatively charged; so also are the particles of organic dispersions, such as milk, charged. Work on protoplasm indicates that the sign of the charge on microscopic particles in living matter is likewise negative. Ultramicroscopic protoplasmic particles appear to be of three kinds, positive, negative, and neutral [218].

Lillie [123] and Gray [85] have observed the migration of spermatozoa of sea-urchins. The direction is to the anode. Lillie also found red corpuscles of the frog negative, migrating at 120 μ a minute; but he found that while the small white corpuscles are strongly negative (1500 μ a minute), the medium sized ones are neutral, or slightly positive or negative, and the large leucocytes positive (120 μ a minute). More recent research by Coulter [42] and others, suggests that the differences observed by Lillie in the sign of the charge of the white blood cell may be due to degrees of acidity. The small negative leucocytes are probably more alkaline and the larger positive cells more acid.

Coulter [42] found the isoelectric point, the point at which no migration occurs, of red blood cells to be at pH 4.6. The corpuscles combine with inorganic ions, and the isoelectric point is the turning point for this behavior. On the acid side the cells combine in much larger amount with the H and Cl ions, while on the alkaline side they combine in greater amount with a cation (Ba).

Falk and Reed [61] from studies of irradiation of blood circulating through a quartz tube inserted in an artery in dogs under ether anesthesia, have found that light applied directly to circulating blood causes a decrease in the negative electrophoretic potential of the red corpuscle.

Some interesting work has been done in an attempt to ascertain if a specificity in electric charge of blood corpuscles exists among animals. Kozawa [114] found that genera of animals are characterized by a definite interfacial potential of the blood, as determined by the concentration of H-ions necessary to make the blood isoelectric. The series obtained was squirrel > guinea-pig > horse, cat, man > dog > goat > sheep > ox, pig. Other investigators find somewhat modified series but definite specific differences are always to be observed. Thus Bernardi [6] obtains the following order based on rate of cataphoretic migration of blood corpuscles: pig > man > guinea-pig > cat > ox > frog > toad > rabbit.

The pathological significance of electrophoretic properties of living cells is evident from the work of Winslow [243], Falk [59, 60] and collaborators on bacteria. Falk, Gussin, and Jacobson [59] find that virulence is fundamentally related to electrophoretic potential. From determinations on 76 strains of

pneumococci isolated from cases of lobar pneumonia, it appears that a definite electrophoretic potential is characteristic of each of the 4 known types; that the type 3 bacterium is of the highest potential migrating at 11 μ per second, thus having an interfacial potential of 14 millivolts. This is two or more times as high as the other three types. The most remarkable feature of this work is the discovery that the sequence of decreasing potential is the same as that of decreasing virulence of the 4 types (of pneumococci for mice) namely, 3, 1, 2, 4. This striking relationship between virulence and interfacial potential of bacteria will undoubtedly lead to fundamental findings in medical physiology. Evidence of this is already forthcoming. It has been shown [60] that the mortality rate among 720 cases of lobar pneumonia decreases from 44.3 per cent to 13.2 per cent with the type of pneumococci in the order 3, 1, 2, 4, which is the order of decreasing potential.

Mudd [154] has demonstrated that the forces involved in the movement of suspended particles in an electric field can transfer living microörganisms across a filtration membrane having certain features in common with capillary spaces in living tissues. Knowledge of the mechanism (presumably electro-endosmotic) of the passage of bacteria through mammalian serous membranes [153] is of fundamental importance in medicine. On such work does our understanding and control of infection rest.

In electroendosmosis possibly lies also the explanation of gland secretion [154], that extraordinary natural phenomenon which seemingly breaks physical laws in that continual diffusion takes place from a region of low to one of high concentration. Concentration is not necessarily the deciding factor. The diffusion is from a region of high free energy to one of low free energy.

Electrophoretic wandering of protoplasmic particles, microscopic and colloidal, has been studied by relatively few investigators. Meier [145] and others have determined the sign of the charge. Meier observed migration of cytoplasmic and nuclear particles toward the positive pole. The most recent work on the subject is that of Taylor [218] who has demonstrated electrical wanderings of microscopic and ultramicroscopic particles in the protoplasm of the slime-mould *Stemonitis*. A direct current of 0.02 amperes between microelectrodes causes migration of the small microscopically visible protoplasmic particles. Ultramicroscopic particles under the influence of a current of 4×10^{-6} amperes move some to the anode, some to the cathode, and some not at all.

If one may indulge in speculation without experimentation, protoplasmic streaming, that little understood continuous flowing of protoplasm, may well be an electrophoretic phenomenon, especially where the streaming is at one time in one direction only, as in the filaments of bread-mould.

Particles which migrate in an electric field, whether blood corpuscles, bacteria, oil droplets, or gold, must do so in virtue of an electric charge. This charge is the outcome of a layer of adsorbed ions. The nature of this layer, whether a double "Helmholtz" layer about one ten-millionth of a centimeter or 1 Å thick, or a cloud of many ions 100 or 1000 Å in thickness, is one of the present-day problems of electrophysical-chemistry [142]. Though the theory of electrophoresis is based essentially on Helmholtz's ideas, the Helmholtz [97] double layer in its original form is by many workers now fully discarded. Helmholtz showed the following relationship between the interfacial potential and the velocity of migration of the particle in an electric field, to exist:

$$v = \frac{\zeta . K . H}{4\pi\eta}$$

where v is the velocity, ζ the potential difference of the double layer, K the dielectric constant of the medium, H the applied electric field (fall of potential within the solution) in volt/cm., and η the viscosity of the liquid. All quantities can be experimentally measured except ζ which can be calculated.

Two layers of an equal number of cations and anions, as Helmholtz assumed, would not give a particle a plus or minus charge but would leave it a neutral condenser. Billiter [10] was the first to object to the Helmholtz hypothesis on these grounds and to suggest an ionic layer of greater thickness. He stated that only when the opposite charges of the two layers are unequal can cataphoretic and allied phenomena occur. Smoluchowski [207] finds the objection unwarranted and points out that cataphoretic rate is independent of colloidal size, a fact which stands in opposition to Billiter's objections.

Electrophoretic wandering of particles which possess equal numbers of oppositely charged ions is intelligible on the assumption that there is a slip between the two layers when an electric current is passed through the system, the inner layer remaining adhered to the moving particle and giving to it its characteristic charge, while the outer layer is repeatedly stripped off and is therefore stationary, remaining a part of the dispersion medium.

This interpretation is apparently not satisfactory to some, with the result that the theory of a cloud or cushion of ions near the surface of the particle, has been advanced in various forms by Gouy [83], Mukherjee [156], Uscher [225], and McBain [142] to remedy the supposed defect of the Helmholtz hypothesis. The objectors to the Helmholtz double layer will also have as their task to account for the movement of ions in a pure salt solution: the ions are always balanced when there is no current.

Pauli [177] in his theory of the chemical complexity and origin of charge on colloidal particles as applied to gold sols, considers the particles as covered with a surface layer of "ionogenic gold complex," the dissociation of which gives rise to the negative charge.

Höber [100] considers the possibility of the selective permeability of the blood corpuscle membrane for anions being responsible for the negative charge of red blood cells, but shows that this alone cannot be true. It would seem that the electric charge on red corpuscles is more likely due to selective adsorption of ions on the surface rather than to selective permeability.

It is very probable that the factors responsible for the electrostatic charge on a particle of gold or a droplet of liquid in an aqueous medium, are also those which endow cells, such as bacteria, blood corpuscles, and algae, and microscopic and ultramicroscopic particles in protoplasm, with electrophoretic properties.

In any complete discussion of the nature of the charge of colloidal particles, the important distinction between the (Helmholtz) electrokinetic potential operative in electrokinetic phenomena, and the (Nernst) thermodynamic potential between the liquid and the solid as a whole—a distinction repeatedly emphasized by Freundlich [73] and his collaborators—should not be overlooked.*

Isoelectric point. Experiments on electrophoretic phenomena, on coagulation and agglutination, have yielded many data bearing on the so-called

* See paper by Leonor Michaelis in Vol. I of this series, entitled "Electric Phenomena in Colloid Chemistry." *J. A.*

isoelectric point of suspensions of living cells and other organic dispersions.

The isoelectric point was first determined for gelatin by Michaelis [149]. His method was that of cataphoresis, and the isoelectric point was given in terms of pH (H-ion concentration). He defined the isoelectric point as that point at which there is a reversal in sign of charge, or, the point of electric neutrality. The pH of this point for gelatin was given as 4.7.

The isoelectric point is seldom if ever a point and often a broad zone. Investigators have made determinations of maximum and minimum values of various physical properties of dispersions and called the point at which these values exist the isoelectric point. It may be that the point of coagulation (of blood), of agglutination (of bacteria), of maximum turbidity (of colloidal solutions), etc., are the isoelectric points of these suspensions, but the actual property studied is not the interfacial potential but some other property which, likely, is at its maximum or minimum value at the same pH at which the P.D. of the suspended particles equals or closely approaches zero.*

The isoelectric point of blood, that is, the H-ion concentration at which blood coagulates, is about pH 4.6. This value is greatly influenced by methods of handling. Washing of the blood considerably augments the negative charge. The pH and therefore the isoelectric point, is lowered, which is probably to be accounted for by the balance of protein and lecithin surrounding blood corpuscles. A lecithin emulsion is at minimum stability at pH 2.3. This value is raised to 4.7 on the addition of albumin and to 5.3 on the addition of globulin [100].

These apparently purely theoretical facts again find practical application in medicine. Fahraeus [58] has determined that sedimentation of corpuscles is three times as fast in women as in men, and is at its maximum (100 times normal) in women at the time of pregnancy. This is very likely due to the magnitude of the interfacial potential, as the increased concentration of lanthanum necessary to cause sedimentation suggests.

Agglutination of bacteria. The stability of bacterial suspensions has been the subject of quite an important and special field of research. Northrop and collaborators have found a definite relation between charge and rate of agglutination. Bacterial suspensions having the lowest charge agglutinate most rapidly. Suspensions having a potential greater than 15 millivolts do not agglutinate at any time.

The following data from Northrop and De Kruif [165] show the relation between the potential difference and the rate of agglutination.

Concentration of Egg Albumin in Per Cent	Mm. per Hour	Potential Difference
0	— 7.5	— 34—never a perceptible agglutination.
0.001	— 4.0	— 18—a perceptible agglutination in 24 hrs. which is never complete.
0.01	0	0—a perceptible agglutination in 1 hr., complete in 4 hrs.
0.1	+ 2.0	+ 9—a perceptible agglutination in 4 hrs., complete in 24 hrs.

Powis [181] and Ellis [53] obtained similar results with oil emulsions, finding them least stable near the isoelectric point. Coalescence occurs below

* See E. O. Kraemer, 4th Colloid Symposium Monograph (Chem. Cat. Co., 1926), pp. 102-121. *J. A.*

a minimum critical value (of about 30 millivolts). Bacterial suspensions are, consequently, similar in electrical properties to emulsions and to metallic suspensions. The latter become less stable as they near the isoelectric point [27].

The importance to medicine of work on the agglutination of pathological bacteria is evident. The effect of antitoxins and immunity serums is probably one of agglutination of the disease bacteria.

While any all-inclusive statement on the relationship between life and electricity would at present be premature, it seems not improbable that the living substance is charged with electrical energy. Investigations so far conducted clearly indicate this. Certainly this energy must manifest itself in various vital processes.*

Permeability

No field of biological endeavor has been more thoroughly investigated and yet yielded so few conclusive and corroborative results than that of permeability. Protoplasm has been humorously defined as a colloid which breaks all the rules of physics and chemistry. The author of this definition undoubtedly had in mind the difficulties involved in experimentation, and the contradictions existing in data and deductions on the permeability of the plasma membrane. Cell permeability is a very large subject and much has been written about it. In a brief summary such as the present, one can mention only a few of the most likely theories and point out some of the problems involved.

A plant growing in the soil may have accessible to it a great variety of elements, but only relatively few of these are found in plant tissues. Some, therefore, gain entrance into the plant and others do not. This property of the cell to select its molecules and ions has long been known as "semipermeability." "Selective" or "differential" permeability would be a more precise term.

How, then, does a cell take in K and all but exclude Na; admit Ca and close the door on Ba? Many cells (red corpuscles, muscles, algae) are rich in K but almost free from Na though the surrounding fluid (for example, the blood plasma) is rich in Na and poor in K. With Ca, the situation is different in different species. Corpuscles contain thirteen times as much Ca as does the plasma, while algae often contain less Ca than the surrounding sea-water. In the case of K the concentration within algal cells may be far in excess of the concentration without, yet diffusion still continues, apparently from a region of low concentration in the surrounding medium to a region of high concentration in the cell. (Again it should be pointed out that concentration is not a force but a result of forces. Concentration may or may not indicate an equilibrium in the forces of which it is a consequent.) The one-sided permeability of water through the intestine membrane which, like the K in algae and corpuscles, presumably passes in one direction more easily than in the other; the more ready entrance of certain substances than others into the cell, NaCl more so than Na_2SO_4, and dextrose more so than sucrose; the high concentration of nectar in plant nectaries secreted from glands, and the similar case of the secretion of substances in high concentration by the salivary glands and the glands of the alimentary tract, are all unsolved riddles of permeability. Equally baffling is the constant change in permeability which the protoplast undergoes. These changes may be occasioned by external

* See papers by I. S. Falk in this volume. *J. A.*

factors, or stand in correlation with environmental conditions as does the difference in permeability of certain plant cells in summer and in winter [84], or they may appear to be entirely controlled (initiated) from within.

Of fundamental importance is the relation between selective permeability and the needs of organisms. Among ions in many respects so similar as are Ca, Sr, and Ba, only the one, Ca, enters the cell in great quantity, and this one is of prime significance for higher green plants in making other ions physiologically available to the plant [224].

That both inorganic and organic substances pass through the plasma membrane, is clear from the fact that plants require inorganic raw material, salts, with which to carry on metabolic processes, and that synthesized organic substances are redistributed in the plant as food. Any theory of permeability must, therefore, account for the passage of both kinds of substances. This is all the more difficult if we accept the hypothesis that salts pass through the protoplasmic membrane by a process different from that of organic compounds.

Organic substances in their passage into cells appear to follow the law of diffusion, that is, the rate is proportional to external concentration. This is apparently not true of salts. Further, the latter enter cells often much more slowly than do organic compounds. Not only does the passage of salts through living membranes sometimes apparently run counter to the law of diffusion in that the internal concentration is often higher than the external, but in ions so closely related as are Na and K the ratio of external to internal concentration may be different.

Jacobs [107] has pointed out that polarity seems to be a determining factor in the differential permeability of salts and organic substances. Salts are polar, highly reactive, ionize readily, have a high dielectric constant, are more readily water-soluble, and enter the living cell slowly. The organic substances, using the hydrocarbons as typical representatives, are throughout the reverse of salts as above described. How far polarity and the other physical-chemical properties of the hydrocarbons are actually responsible for the more rapid entrance of these substances into the cell, is an as yet unanswered question.

The classical theories of permeability, in spite of much buffeting about by adverse criticism, still meet the demands of experimental data as well as do more modern hypotheses. This is true of the much maligned theory of Overton [173, 174] based on the most intensive piece of research yet done on cell permeability. Overton found that those substances which penetrate the cell most rapidly, methane, xylene, alcohol, etc., etc., dissolve fats readily. He believed, therefore, that the plasma membrane is principally lipoid in nature. There has never appeared in the work of others [13, 110] any exception to the rule that fat-soluble substances enter the cell with great rapidity. This is true, for example, of the glucosides, saponin and similacin [200].

A severe criticism of Overton has been that he neglected the very evidently necessary passage of salts and other water-soluble nutrient substances. This he did not do. His policy in never replying to attacks has allowed this and other adverse criticism to stand unchallenged. Overton did not regard the plasma membrane as made up wholly of fats. He believed it to be chiefly lipoid. He realized that his theory did not explain the entrance of water-soluble substances but he was not primarily concerned with this phase of the problem. He sought an explanation of his observations, namely, that fat-dissolving substances enter with extreme rapidity, much more quickly

than do water-soluble substances. Actually, it appears that equilibrium in salt concentrations within and without the cell is never reached in no matter how long a time (Brooks [20] has shown that the concentration of arsenic in protoplasm never equals that of the external solution until death takes place), while equilibrium in alcohol concentrations, for example, is attained in a very few seconds. These differences are facts of fundamental importance, and to Overton is their discovery due.

Parchment paper impregnated with lecithin and used as a membrane to separate pure water from a solution of chloride, permits the salt to pass through the membrane. This experiment lends strong support to Overton's hypothesis, for such a membrane being fatty in nature will permit the rapid passage of ether, chloroform, etc., and at the same time, as the experiment proves, let salts in solution through.

Great as were Overton's contributions it is nevertheless true that the lipoid hypothesis does not satisfy all membrane diffusion phenomenon. Further, there is much evidence to show that the plasma membrane is, in part, protein in nature.

Traube [223] and Czapek [44] regarded the mechanism of entrance of substances into the cell as a surface tension phenomenon. The latter believed that a substance must have a tension value less than 0.685, which he thought to be the surface tension of protoplasm, in order to pass through the plasma membrane. His theory met with drastic criticism [212] to which Czapek [45] made the reply that the theory must collapse only when one substance has been found which in spite of a surface tension value below 0.685 is not deadly.

Warburg [231] has, in the main, discarded all lipoid and surface tension hypotheses for one of adsorption. He finds that the degree of toxicity of the methyl, ethyl, propyl series is much more closely related to the adsorption powers of these alcohols than to the surface tension or lipoid solubility values.

Raber [184] has advanced an "electrostatic" theory of membrane permeability. He believes the entrance of a salt into a cell is determined by the sign and magnitude of the electrostatic charge on the colloidal elements of the plasma membrane and by the charge on the ions of the penetrating salt. The protoplasmic particles of the membrane are assumed to be negatively charged, which has support from the work of Heilbrunn [94] who claims to have shown that the particles in the interior of the cell are positively charged while those at the surface are negative.

That the effects of salts upon permeability is due to the interaction of the charges on the protoplasmic particles and on the salt ions—increased permeability being the result of peptization and decreased permeability of coagulation—has been suggested by a number of workers, notably Spek [210], Kahho [111] and Fenn [62]. Until more evidence on the precise electrical nature of protoplasmic particles is available, conclusions as to the validity of permeability theories based on charge must be held in abeyance; yet it does appear that such theories give much promise of an explanation of protoplasmic permeability.

An emulsion hypothesis of the mechanism of permeability is that of Clowes [40] who found in the behavior of emulsions a striking analogy of the experimental data of Osterhout [170] in his work on the permeability of the plant cell. Osterhout found that NaCl increases and CaCl (at first) decreases permeability. Loeb [133] had previously found an "antagonism" between Na and Ca, that is, Na and Ca when present in proper proportions prevent the toxic effect of each other; this proportion is that which exists in any

"physiologically balanced" solution (sea-water, blood, milk). Clowes found that Na hydroxide produced water and oil emulsions of the oil-in-water type while Ca caused reversal to the water-in-oil type. NaCl and CaCl₂ in the proportion of 100 molecules of the former to one or two of the latter, which is approximately the proportion in which these salts occur in sea-water and in blood, balance one another. The mixture has no effect on the emulsions. Clowes, with some justification if protoplasm is to be regarded as an emulsion, viewed the plasma membrane as a system in which oil is dispersed in water when the membrane is more permeable, the condition which Na produces, and as a system of water dispersed in oil when the membrane is less permeable, a condition produced by Ca. Normally, the surface emulsion which constitutes the protoplasmic membrane, is in a state of equilibrium near the reversal point, since it is bathed in a balanced solution. It is, therefore, readily thrown one way or the other by a change in concentration of the salts in the surrounding medium.

Clowes' hypothesis is a very ingenious one and gives an ideally simple explanation of what in all its aspects appears to be a very complex phenomenon, but since NaCl which alters protoplasmic permeability, will not reverse the oil emulsion, and since when certain other stabilizing agents (albumin, etc.) are used instead of that (soap) employed by Clowes, none of the salts will reverse the emulsion, and since the ultimate structure of protoplasm is in part, and primarily, that of a protein, the hypothesis cannot be regarded as proved. However, if the living plasma membrane is coated with an oil film, we must admit that fatty substances, and therefore, possibly, emulsions, determining, in part, the entrance of substances into cells.

The literature on the effect of ions on protoplasmic permeability is prolific and the results very contradictory. It is rather generally believed that salts of monovalent cations increase permeability while salts of bivalent cations decrease permeability. Loeb [132], Osterhout [170], Scarth [192], Chambers and Reznikoff [34] and others have found that the monovalent cations Na and K have a like (diminishing) effect upon the permeability and consistency of protoplasm, while the bivalent cations, Ca, Ba, and Mg have just the opposite influence. Loeb [135] found the same to be true of the effect of these ions on proteins. An experiment (by Michaelis) illustrates the influence of one of these salts. Apple skin contains much potassium. None of this escapes into water within a week, but if the apple is soaked in 0.1 N NaCl a trace of K is evident in 3 days, 0.01 mg. in 5 days, and 0.12 mg. in 11 days.

That NaCl increases protoplasmic permeability is the generally accepted opinion. The same is held for other monovalent cations, yet Fitting [65] obtained marked lowered permeability of the cells of *Rhoeo* with KNO₃ and salts of other monovalent cations. Brooks [23] obtained similar results with the alga *Nitella*. All the salts studied (NaCl, KCl, CaCl₂, MgCl₂) decrease the rate of penetration of the dye (Dahlia), but the bivalent ones are more effective.

There has been a strong tendency in the past for chemists and biologists to group ions on the basis of valency in the belief that all of one valency have a like effect on protoplasm and on proteins. The above cited work of Fitting and Brooks tends to show that such a valency grouping is not sound. Seifriz [200] also finds marked differences in the reaction of protoplasm to the two bivalent cations Ca and Sr. In the leaf cells of the water-plant *Elodea,* Ca decreases permeability, Sr increases it; Ca increases viscosity, Sr decreases

it; Ca has no effect on protoplasmic streaming, Sr greatly accelerates it. On the basis of a physiological interpretation it is to be expected that protoplasm should react differently to the two elements Ca and Sr. Physico-chemically, perhaps, the two bivalent cations should have a like effect. For the biologist, however, the experimental results are in harmony with other known fundamental facts. Plants thrive on Ca but not on Sr which is toxic to them. Ca occurs regularly and in considerable quantity in all plant tissues, Sr not.

L. Loeb et al. [137] found that all salts of potassium usually increase the normal consistency of amoebocytes, but this alteration may deviate to the side of increased fluidity depending upon the concentration of salts and on the osmotic pressure of the solutions used. Scarth [194] finds that the permeability of living cells of Spirogyra for acid dyes increases with the osmotic pressure of the medium in which the dye is applied.*

The valency grouping is further weakened by the emphasis of several workers that the opposite effect of NaCl and $CaCl_2$ on protoplasmic permeability, and the "antagonistic" action of these salts in a physiologically balanced solution, is not due to opposing effects of, or antagonism between, the two cations, but, as Clowes [40] states, between cations on the one hand and anions on the other, Fenn [62], from work on gelatin, comes to a similar conclusion, namely, that anions antagonize cations. Kahho [111] expresses essentially the same opinion when he says that the action of a salt on the plasma membrane is the additive effect of the opposite acting ions; cations have a coagulative effect and lower permeability while anions have a peptizing or dissolving action and increase permeability.

In a fundamental modification of all his earlier work on the effect of Na and Ca on the eggs of the fish *Fundulus,* Loeb [134] makes the following qualification to the then generally held belief that Na and Ca have opposite effects, i.e., that elements of different valencies have different influences on protoplasm. Loeb states that in moderate concentrations NaCl acts exactly like $CaCl_2$ or $LaCl_3$, each retarding the rate of diffusion of acid—the minimal concentration of the salt in which this inhibiting effect occurs diminishes rapidly with the valency of the cation. Furthermore, while the chlorides of Na and Ca (and La) inhibit the diffusion of acids through the membrane, both NaCl and $CaCl_2$ accelerate the diffusion of dissociated strong alkali. To recapitulate: Na and Ca have an opposite effect on protoplasm at higher concentrations, but at lower concentrations they behave similarly, yet while alike in their influence they inhibit the diffusion of acids but accelerate the diffusion of alkali.

The factors involved in cell permeability are many and until they are known general deductions are likely to be highly speculative.

Methods [84] of measuring protoplasmic permeability are, in part, the cause of the unsatisfactory state of the subject. Neither the older plasmolytic method [200] nor the newer conductivity method [26] is fully dependable. Differences in protoplasmic behavior are certainly also in great measure responsible for discrepancies in data. There is no reason why all masses of protoplasm or even the same mass at all times, should react the same to a salt. A pine tree and an amoeba are different in every aspect of their behavior; why assume that their protoplasm will react similarly to environmental stimuli, or is the same in its physical properties? Certain fundamental similarities

* See paper by Leo Loeb in this volume, *J. A.*

do exist—thus, all protoplasm so far studied is elastic—but the differences must be excessively greater than the similarities. This fact as much as method may be responsible for Brooks [23] finding that NaCl decreases the permeability of the fresh-water alga *Nitella* and for Fitting [66] finding that KCl decreases the permeability of the land-plant *Rhoeo* while Osterhout [170] finds that this salt increases the permeability of the sea-weed *Laminaria*.

There is further the question of acidity of the surrounding medium. Brooks [24] finds that the rate of penetration of arsenic is influenced by changes in external pH. The H-ion concentration of the soil solution differs, considerably in different localities and must at different times vary in the soil of any one plant. With change in acidity occur differences in permeability to salts.

Mild application of narcotics, in general, increase protoplasmic permeability. This has been shown to be true for ether by Höfler and Weber [101]. The same is true of ethyl alcohol [200]. The importance of the effect of anesthetics on cell permeability is beginning to be appreciated in the medical profession in connection with such problems as postoperative psychosis [143].

There is a possible correlation between permeability and viscosity. Applications of narcotics in low concentrations increase permeability and decrease viscosity. Nadson and Meish [157] demonstrated that chloroform accelerates protoplasmic movements and diminishes viscosity. Numerous workers [235] have shown that ether, alcohol, chloroform, etc., decrease protoplasmic viscosity. High concentrations of narcotics or prolonged applications of low concentrations produce the opposite effect, ultimately causing irreversible coagulation and death.

Bersa [7] and Dixon and Bennett-Clark [49] find that electric currents cause changes in permeability.

An interesting and important question, still in the controversial stage—as, indeed, is the entire subject of permeability—is that having to do with the relative ease of entrance of ions and molecules into the protoplasm.

Jacobs [106] believes to have shown that CO_2 enters cells in the form of the undissociated molecule. Following up Jacobs' discovery, Osterhout [171] claims that only undissociated molecules penetrate a living cell. He bases this conclusion on the observed behavior of the alga *Valonia* in the presence of solutions of CO_2 and of H_2S. The data show that the amount of CO_2 or H_2S in the sap of the vacuole is proportional to the amount of undissociated CO_2 or H_2S in the solution bathing the cell.

First, as regards carbon dioxide, the plant does not have the CO_2 molecule as such to deal with. When CO_2 enters water and dissolves, carbonic acid, H_2CO_3, is formed which is partly dissociated, and, if older ideas still hold, in two stages: $H^+HCO_3^-$, and then to $H^+H^+CO_3^{--}$. Since CO_2 cannot get into the cell except through water, the cell can have only the H_2CO_3 molecule and the ions H^+, HCO_3^- and CO_3^{--} to deal with.

Second, any general theory on the relative ease with which ions and molecules enter cells will have to conform with present day conceptions on dissociation. The work of Bjerrum [12] and of Debye [47] has greatly modified older ideas on the degree of dissociation of electrolytes.

The plant has to deal with concentrations of 1/100 to 1/1000 molar or less. Elements taken up by the plant are present in the soil in concentrations from 0.0001 to 0.013 equivalents per liter [221]. It has, therefore, in the

past, been assumed that such concentrations of electrolytes leave a fair proportion of the salts in the undissociated state; thus, calculations from equivalent conductance data in the Landolt-Börnstein [130] tables give the following values, in percentage of undissociated salts; at 0.0001 M. concentration, $KCl = 0.8$, $NaNO_3 = 0.7$, $\frac{1}{2}CaSO_4 = $ ca. 1.0; at 0.01 M. concentration, $KCl = 5.9$, $NaNO_3 = 6.8$, $KH_2PO_4 = 7.0$, $\frac{1}{2}CaSO_4 = 33.0$.

The epoch-making research of Arrhenius on the dissociation of electrolytes has been found to be incomplete. The Arrhenius and Nernst formulas do not measure the degree of ionization as formerly believed, but they measure how much the electric fields in the solution interfere with the solubility of the ions.

That strong electrolytes are fully dissociated was early expressed by Bjerrum [12] who gave in a brief and most precise thesis the essential facts of the whole problem. He states that strong electrolytes always are completely dissociated into ions. The unexpected decrease in molecular conductivity and in molecular depression of the freezing point that accompanies increase in concentration is due not to incomplete dissociation but to electrolytic friction, that is, to the action of the electric charges of the ions on each other. Molecular conductivity is diminished not because the number of ions is decreased but because the ions move more slowly.

According to Bragg and Bragg [17] NaCl is completely ionized in the solid crystalline state. How, therefore, can it be incompletely ionized in the dissolved state?

We seem, therefore, to have nothing left in the solution of a strong electrolyte which could be thought of as an undissociated molecule, except the possible presence of electrically neutral doublets concerning the nature of which little is known but to which we shall have to resort to find at least a partial escape from our difficulties.

With weak electrolytes the state of affairs is different. There is little dissociation, so that molecules are present in abundance in solution. Bodine [14] and Brinley [19] suggest that HCN enters the cell as a molecule and ionizes within the cell (to account for an intercellular acidity when the extracellular HCN solution is slightly alkaline). KCN is likewise thought to enter the cell in the molecular state but as HCN. The KCN hydrolyzes $(KCN \rightleftarrows H_2O \rightleftarrows HCN + K^+OH^-)$ into weak electrolytic HCN which enters the cell as a molecule.

Brooks [20] objects to any all-inclusive theory that only molecules enter cells, since she finds no relation between the amount of arsenic in the sap of *Valonia* and the amount of undissociated molecules of arsenic in the surrounding solution.

Fat-dissolving non-electrolytic substances, such as the alcohols, glucosides, ether, chloroform, etc., for which the cell is rapidly permeable, seem to enter in the molecular state, but salts, of which every living thing has need, must enter in the ionic state, the only state in which they exist in solution.

The problem appears to be not whether ions *or* molecules enter the cell, but how do ions and molecules *both* pass through the membrane.

That membranes are apparently permeable only to the cation or anion of a salt has been mentioned in the discussion of membrane potentials, and in this connection was considered the problem of how one or the other kind of ion can pass alone through a membrane. It was shown that this is not possible unless we give the diffusing ion another mate. It was further shown that all

difficulties are best satisfied by the electrostatic theory of permeability advanced by Raber [184], Michaelis [149], and others. This hypothesis is, however, incomplete since the mechanism of the entrance of non-electrolytes cannot be electrostatic.

A word of caution should be said regarding certain postulates as to the presence of pores in membranes. For many years the protoplasmic surface has been compared to a sieve. A sieve mechanism of permeability has recently been advanced in an interpretation of membrane potentials [149]—it was discussed under membrane potentials. It may be that collodion and other types of precipitation and gel membranes are porous in structure, though the evidence in support of this is not conclusive. In the following discussion on protoplasmic structure it will be seen that gelatin, proteins in general, and protoplasm are regarded as built up of linear structural units of possibly molecular dimensions (chains of amino-acids), and that micellae or like units, and therefore pores of colloidal size, are not present. If by "pores," in the protoplasmic membrane, is meant the interatomic spaces of the protein jelly, then the pore theory can still stand.

All possibilities as to the nature of the protoplasmic membrane are to be found in recent hypotheses. There is the one extreme of no membrane, which likens protoplasm unto a block of gelatin whose surface (if freshly cut) may not differ from the interior. The identity of living matter, its immiscibility in water, are undoubtedly primarily due to continuity in structure, as in the case of gelatin, rather than to the presence of a membrane; yet there is every evidence, theoretical and experimental, to support the existence of a morphological membrane surrounding all protoplasts. The simplest possible surface film is a monomolecular layer. Fricke [77] is of the opinion that the layer determining permeability is one molecule thick, even though the pellicle *in toto* is thicker.

The presence of an optically visible membrane on certain protoplasts and nuclei has been conclusively demonstrated [105, 199]. The question no longer is, Is there a membrane on protoplasts? but, What is the physical and chemical nature of this membrane? The work of Overton [173], Mudd [155] and others, points very convincingly to an outer oil film behind which is the morphological membrane whose presence is sometimes anatomically demonstrable [105, 109]. The high elastic properties of the membrane indicate clearly an essentially protein character. The framework of this protein membrane, like the protoplast as a whole, is permeated by crystalline and fatty substances in aqueous dispersion.

THE HOFMEISTER SERIES

An interesting and important controversy has waged around the so-called Hofmeister series of ions. Hofmeister [102], on the basis of the relative effects of anions on the salting out of egg albumin, obtained the following series: citrate > tartrate > sulphate > acetate > chloride > nitrate > chlorate. Similar lyotropic series have since been obtained by others. Thus Pauli [176] gives the following order for the relative efficiency of acids on the viscosity of blood albumin: hydrochloric > monochloracetic > oxalic > dichloracetic > citric > acetic > sulfuric > trichloracetic. The strong monobasic acid, hydrochloric, increases the viscosity of blood most. It is followed by the weak monochloracetic acid and this by the dibasic oxalic. Later comes the weak tribasic citric acid which is followed by the weak monobasic acetic,

and this, in turn by the strong dibasic sulfuric which joins another monobasic acid, trichloracetic. All such series show no relation to the stoichiometrical properties of the ions. The Hofmeister and related series of ions have been attributed to an experimental error by several workers, notably Loeb [135]. It is believed that what is actually an effect of an alteration of the H-ion concentration has been erroneously attributed to a specific action of the anion; that only the valency and not the other properties of the anion of an acid influences the swelling and viscosity of gelatin; that the Hofmeister series of salt effects are purely fictitious; and that the influence of acids on the physical properties of gelatin is due to the Donnan equilibrium between the protein solutions and the surrounding aqueous medium [50].

This view-point has gained wide favor, but more and more is there a tendency to return to the older idea that each ion has its specific action. It is rather extraordinary that the contrary should ever have been assumed to be true. Undoubtedly the relative effect of ions in certain systems is due, in part, to the change in H-ion concentration which they occasion, but certainly also, in part, if not primarily, to their chemical specificity.

Loeb [134] corrected, as has been stated, his earlier valency grouping of cations in regard to their effect on protoplasmic permeability, since low concentrations of Na, Ca, and La all inhibit the rate of diffusion of acid. Work by Klobusitzky [113] on the speed of settling of erythrocytes and on blood coagulation indicates that in relative effect on decrease in time of blood clotting, the salts of K form the following Hofmeister series: $SO_4 < Cl < NO_3 < Br < I < SCN$. One assumes, of course, that pH is kept constant, otherwise the experiments are meaningless. This series closely parallels that of Lillie [124], done before the days of pH control, on the depressing effect on the osmotic pressure of gelatin solution.

Scarth [193] in his studies on the elasticity of gelatin, finds that the reciprocal of extension (resistance to stretch) has a minimum value at pH 4.7 and maxima at pH 3 and pH 11. He also finds that a salt with a trivalent (i.e., colloidally active) cation has the same effect at a uniform pH (of 4.7) as has the addition of acid or alkali to isoelectric gelatin. These data support the above contention that both pH and ion specificity are factors influencing the physical properties of gelatin.

Among the latest investigations on the influence of electrolytes on colloidal systems, involving very careful control of pH, are those of Skeen [206] who worked on the lyophobe kaolin. These results tell nothing about the salt effects on lyophiles (gelatin and protoplasm) but they do indicate that on one type of system pH is not the sole determining factor. Skeen used as criteria, the turbidities produced by suspended kaolin, the final volumes of sedimented kaolin, conductivity, pH, and intensity of charge on the particles. He found a correlation between degree of turbidity and volume of sedimented kaolin but the degree of turbidity is independent of pH, and there is no correlation between sign or intensity of charge and turbidity or final volume of sediment. Further, pH does not control the degree of adsorption of ions, nor is the effect of adsorbed electrolytes on flocculation due to pH, but to specific effects of the ions. HCl is adsorbed at all concentrations, but other electrolytes used are not. The following adsorption sequence is obtained: $AlCl_3 > CaCl_2$, $BaCl_2 > NaOH$, NaCl. In reversing the sign of the particles, the following sequence results: Al > HCl > Ca > Ba. Al was found to be the only ion to effectively flocculate kaolin.

The Protoplasmic Membrane

Theories of protoplasmic permeability have, for the most part, postulated a surface membrane. Some there are who deny the existence of such a membrane. Fischer [64] says there are no membranes around cells. Micro-anatomical evidence disproves this, but Fischer is right in implying that the surface is, in the main, the same type of system as is the inner protoplasm.

In tearing a coagulated mass of protoplasm it is always the surface which tears last, indicating a higher tensile strength of the outer layer than that possessed by the inner protoplasm. Howland [105] gives proof of the presence of a membrane surrounding *Amoeba verrucosa*. It is also possible to lift off (the coagulated) nuclear membrane of amoeba [199]. The work of Chambers and Reznikoff [34] supports the presence of a surface layer which reacts differently to external salt solutions than does the inner protoplasm when the same salts are injected into the cell. Scarth [194] states that experimental facts point to an organized film on the surface of protoplasm as the regulator of permeability.

The work of Howland [105] tends to prove the presence of an outer and an inner protoplasmic membrane. The former is of perceptible thickness, and capable of being lifted off (in the coagulated state). The inner membrane is an infinitely more delicate layer indistinguishable from the protoplasmic mass as a distinct film.

There can be no doubt but that most cells possess definite morphological membranes which may assume the thickness and rigidity of the pellicle of a protozoan (such as *Euplotes*) or be as delicate and imperceptible a layer as that covering leucocytes. Some there are who prefer putting membranes into different categories, and regarding the pellicle as quite distinct from the microscopically invisible surface film. But it appears that all surface coverings on living matter are manifestations of the same innate tendency in protoplasm, namely, to clothe itself with a protective layer.

The surface layer of amoeba is at times a tough elastic membrane which, however, becomes, at the tip of an advancing pseudopodium, quite thin, offering little resistance to a microneedle. This can be readily demonstrated by micrurgical methods [198]; that it must be so is evident to any student of amoeboid movement. A somewhat different case, but one which emphasizes still further that protoplasmic membranes, as tough and rigid as they may be, are still capable of pronounced changes in consistency, is that of the pellicle of Euplotes. The outer layer of this protozoan is normally very resistant, yet when the organism is suffering from a pathological condition (occasioned by mechanical disturbance), huge globular protrusions may form at the surface, not small clear exuded droplets such as are often seen on protozoa, but a massive globule involving the extension of the former rigid but now fluid pellicle, with the granular protoplasm following. In brief, the outer protoplasmic layer is a living intimate part of the protoplast as a whole. To regard the outer membrane in any other light would mean a similar interpretation of the nuclear membrane which (in amoeba) also can be nicely lifted off (in the coagulated state), and it is inconceivable that the nuclear membrane is anything more than a specialized bit of living matter.

Another instance, intermediate between that of Amoebae and Euplotes, is that of amphibian red corpuscles [204]. These may form globular and finger-shaped protrusions involving the actual extension of the erythrocyte membrane.

The membrane of red blood cells is a moderately tough and quite elastic structure which undergoes changes in consistency, one such being that involved in the formation of protrusions at the surface. Evaginations which cause an extension of protoplasmic membranes, are not to be confused, here or elsewhere, with those exudations which appear on the surface of erythrocytes and other cells and do not involve an extrusion of the membrane [204].

From the foregoing it is clear that workers are pretty generally agreed on the presence of a morphological membrane enclosing all protoplasts. The problem now appears to be, not to prove the presence of such a membrane, but to ascertain its nature. The high elastic qualities of the surface layer of protoplasm indicate an essentially protein constitution [203], a protein framework permeated by an aqueous solution of salts, and organic substances.

The amino-acid chains of the protein constituents of protoplasm form the structural framework of the living substance. This structure extends to the surface where a closer interlocking of the fibrous units gives greater rigidity to the outer layer. The denser protein framework at the surface of a protoplast is bathed with an aqueous dispersion relatively rich in fatty substances, just as is the inner protoplasm. According to the Gibbs-Thomson principle these fatty substances will aggregate at the surface where this protoplasmic emulsion is exposed and form there a thin oil film coating the morphological membrane. The thickness of this oil film can only be conjectured. It may be a monomolecular layer or it may be many molecules thick.

The presence of an oil layer on protoplasts would at first thought appear to satisfy the experiments of Overton. Whatever our picture of the protoplasmic membrane is, it must account for the extraordinarily rapid entrance of fat-soluble substances. An oil film coating the morphological protein membrane of protoplasm will account for the marked increase in permeability which fat dissolving substances produce only if the protoplasmic mass itself is readily permeable to these substances. Breaking down the first barrier is simply the initial step toward rapid diffusion throughout the cell which fat solvents accomplish.

An oil film covering the surface of protoplasts must also be permeable to those water-soluble salts which satisfy the needs of the cell. It has been stated that lecithin impregnated parchment paper permits the passage of salts in solution.

The evidence supporting an oil film on the outer surface of the morphological, essentially protein membrane of protoplasts is convincing though not conclusive. There are comparable cases in nature where apparently pure protein membranes surround fat globules. Milk is such a case. Especially instructive are the latex globules of rubber which are covered with protein membranes [112]. To these protein membranes are the (coagulative and elastic) properties of rubber due. There is no evidence of an outer oil film here though the interior is all hydrocarbon.

An oil film coating protoplasts may account for the rapid entrance of fat-dissolving substances and the corresponding low passage of salts, and thus have a marked influence on permeability, but it cannot fully account for all of the extraordinary features of selective permeability. The protein complex which constitutes the framework of the protoplasmic surface is the seat of the more intricate permeability control. No theory of permeability based on a passive membrane can possibly satisfy all permeability phenomena. Freund-

lich [70] points out the weakness of the sieve hypothesis of membrane mechanism by stating that on the basis of sieve action one should be able to arrange membranes in a series in the order of their permeability, but this is by no means the case.

To attempt to visualize the mechanism of permeability control in such a simple way as Overton did with the lipoid membrane or as did Clowes with an oil emulsion, is quite impossible. But it appears probable that the oil film described by the former is *one* of the factors involved, and it is not impossible that emulsions play their part. (There is, however, no evidence to support the presence of water-in-oil emulsions in protoplasm.)

The early but still sound work of Chodat and Boubier [37] leads these workers to the conclusion that the behavior of the protoplasmic membrane is essentially that of proteins.

A new and ingenious method of ascertaining the nature of the outer layer of cells has been employed by Mudd and Mudd [155] who observed the behavior of bacteria, sperm, erythrocytes, etc., when they come into contact with the interfacial film between water, in which the cells are suspended, and oil. The consistency of the cell membrane is indicated by the ease with which the interfacial film distorts the cell. Red corpuscles are pulled into the shape of a lens, indicating high consistency (and elasticity) though the membrane is pliable enough to permit distortion. (Microdissection evidence [204] is in full agreement with this.) Leucocytes when caught in the interfacial film disintegrate quickly. Their membranes cannot resist the tension.

Of value are the data obtained by Mudd and Mudd on the chemical nature of the protoplasmic surface. Erythrocytes are readily wet by oil and pass through the interfacial film into the oil rather easily; after sensitization with anti-erythrocyte serum the red corpuscles are no longer readily wet by oil, probably because of the coating over of their lipoid surface with substances from the immune serum. The interfacial data are consonant with analytical, cataphoretic and titration data in indicating the presence both of protein and of lipoid in the normal red cell surface.

The behavior of bacteria is especially interesting. Non-acid-fast bacteria are not easily wet by oil; they pass into it only at the expense of considerable mechanical work. Acid-fast bacteria (tubercular), on the other hand, when touched by the interfacial (oil-water) film are thrust immediately into the oil. If in clumps, the bacteria may be dispersed explosively by the interfacial stresses and the scattered bacteria appear in the oil phase. The fatty surface material responsible for this behavior of acid-fast bacteria may be extracted by alcohol, leaving a surface which behaves essentially like that of ordinary bacteria, or may be coated over with material from immune serum. Tubercle bacilli maximally sensitized with specific immune serum behave as though coated with denatured serum globulin. It is quite evident that the surface of bacteria differs strikingly in different species.

VISCOSITY

Viscosity values of protoplasm include all possible ones from a very liquid, though never watery, state, to that of a firm resilient jelly. This is, indeed, the expected situation if we regard protoplasm as primarily a colloidal system essentially like any turgescible jelly in its mechanical properties. The water content of protoplasm varies from as low a percentage as 15 to as high as

97. So great a variation in amount of water in an elastic hydrophylic jelly must mean a wide range in consistency.

Ingenious, and often indirect methods have to be devised to determine viscosity values of protoplasm, since the living substance must remain not only alive but normal during the measurements.

The two methods which most readily permit quantitative evaluations of protoplasmic viscosity are the gravitational and the centrifugal. In the former the cell and its contents serve as a gravity viscometer.

Certain cells (of the plant *Vicia faba,* for example) contain starch grains (statoliths) which lie at the bottom of the cell (presumably serving as gravitational sense organs). If a cell is exposed to view and the position of the plant reversed through 180°, the starch grains fall. Their rate of travel is measured and serves as an indicator of the viscosity of the living fluid. The results are purely comparative unless the grains are afterward removed from the cell and their rate of fall in a known standard (water) determined. This has been done by Heilbronn [92], who obtained a specific viscosity value of 23.7 (water = 1) for the protoplasm of the root cell of *Vicia faba.*

Weber [234] has determined the viscosity of cell sap by the gravitation method, using the calcium oxalate crystal in the vacuole of the plant *Callisia* as falling bodies. He obtains, by comparison with the rate of fall of similar isolated crystals in water, a value for cell sap of not quite twice that of water, which is in good agreement with the earlier findings of Ewart [57] by the osmotic method.

The protoplasmic granules in the embryonic cells of root tips of plant seedlings [162] and in the ova of certain marine organisms [94] become stratified when centrifuged. The degree of stratification is an indication of comparative viscosity [162]. So also is the force necessary to cause stratification a measure of viscosity [94]. The centrifuge method has recently been calibrated and a viscosity value of four times that of water found for the protoplasm in the eggs of the clam *Cumingia.* The endoplasm of the protozoan *Paramecium* has a specific viscosity value of 8000 as determined by centrifuging [63].

A novel method for making viscosity determinations of protoplasm was devised by Alfred Heilbronn [93], who inserted metal particles in living protoplasm and attracted them with an electromagnet. The pull necessary to put the particle in motion is a measure of the consistency of the medium. A similar method with some modifications was independently devised, primarily for making elasticity determinations, by Freundlich and Seifriz [76].

An indirect method of making viscosity valuations of protoplasm is that of microdissection or micrurgy [178]. The protoplasm is dissected with mechanically controlled glass needles of great fineness. The method, while permitting but estimations of consistency, is the only one which makes possible even crude valuations of minute regional differences in viscosity of a protoplast, and of cell structures such as nuclei and chromosomes. Chambers [32] * has determined local differences in the consistency of the dividing echinoderm egg. 'Seifriz [198] has made estimations of the viscosity of a variety of plant and animal protoplasm. Chambers and Sands [35] have isolated living chromosomes and found them to be of high "gel" consistency.*

The viscosity of protoplasm may, in different protoplasts and in the same

* See paper by Robert Chambers in this volume. *J. A.*

protoplast under different internal physiological and external environmental conditions, vary from a low viscosity, approaching but never equalling that of water, to the extraordinarily high viscosity of a firm jelly. Failure to understand this fact means failure to appreciate one of the fundamental physical properties of protoplasm.

Marked changes in consistency are coincident with reproduction [129, 198, 238], with streaming activities [198], with pseudopod formation [198], with growth, injury, disease, etc.

Changes in protoplasmic consistency take place in the normal activity of the cell but may also be induced by environmental factors such as temperature, narcotics, electrolytes, etc.

Protoplasmic viscosity falls with rise in temperature within normal limits. There are, apparently, exceptions to the rule that protoplasm is passively amenable to environmental temperature changes as is a solution of gelatin, that is, there appear to be internal regulatory factors. That there is an internal mechanism for controlling temperatures in organisms we know in the case of all warm-blooded animals. In the case of plants the situation is the opposite. The temperature of plants rises and falls with that of the air except at the extremes. (Corn in the field will not go much above 90° F. even though the air temperature reaches 110° F. Cacti are known to have reached 130° F. in the Arizona desert.)

One would expect the viscosity of the blood of warm-blooded animals to remain fairly constant but this is apparently not the case. Burton-Opitz [28] has experimentally demonstrated that blood reacts very sharply to outside influences. Cold baths increase and warm baths decrease the viscosity of the blood of dogs considerably. The same appears to be true of man.

Occasionally, protoplasm seems apparently to run counter to the usual procedure of organic colloids and becomes, for example, less viscous instead of more viscous with fall in temperature. When such events happen the biologist is led to regard the temperature-viscosity relation as a physiological and not a pure physical-chemical one. By this he means that protoplasm does not follow the known physical-chemical laws laid down for such simple systems as gelatin, and that certain reactions going on in protoplasm follow other physical-chemical laws about which we know nothing; or that known laws are modified. If we can interpret the mechanics of it, the reaction is physical-chemical. If we cannot interpret it, we call it, temporarily, physiological.

Weber [236], Lloyd [129], and others have determined changes in the viscosity of protoplasm induced by anesthetics—low concentrations of alcohol and ether decrease, while high concentrations increase viscosity. Jacobs [106] finds the same changes are brought on by CO_2.

Scarth [190] has shown that among electrolytes, salts of the alkaline metals (monovalent) decrease the adhesive qualities of protoplasm, presumably therefore lower viscosity, while salts of the alkaline earths (divalent) and of the rare earths (trivalent) increase adhesion and presumably viscosity.

Chambers and Reznikoff [34] have injected microscopic quantities of salt into living protoplasm (Amoeba) and find that NaCl and KCl liquefy, while $CaCl_2$ and $MgCl_2$ tend to solidify the protoplasm.

These data are in harmony with the widely accepted hypothesis that the monovalent cations tend to lower viscosity and increase permeability, while the bivalent cations have the opposite influence. This effect is also true of proteins [135], and, in part, of emulsions [40] where liquid oil-in-water

systems are produced by NaOH and firm water-in-oil emulsions by CaCl₂.

We have already considered in the discussion on permeability the soundness of the valency grouping of ions as applied to protoplasm. Among other data it was mentioned that J. Loeb [134] found that Na and Ca have a like effect at low concentrations, and L. Loeb found that K salts both increased and decreased protoplasmic consistency depending on the concentration and the osmotic pressure of the solutions.

Bersa and Weber [8] have studied the effect of an electric current on protoplasmic consistency and find that an increase is induced by a current of 5-10 milliamperes.

Studies on protoplasmic consistency while at first thought of purely theoretical interest, have an important bearing on many physiological problems. The dynamics of such vital phenomena as mitosis [162], the functioning of vacuoles [217], amoeboid movement [140, 209], muscular contraction [78], permeability [208], the organization of monomolecular layers in living matter [168] and protoplasmic structure [191], are intimately associated with the viscosity of protoplasm. Lewis [122] emphasizes the importance of the adhesive quality of cells in pointing out that without it multicellular organisms could not exist.

Just what the mechanism of viscosity changes in protoplasm and colloidal jellies in general is, is not fully known. Beyond doubt the structure of the elastic jelly itself is an important factor. Consideration of this will be given on the following pages. Other interpretations are advanced by Alexander [3].

No attempt has here been made to distinguish between viscosity and plasticity. Bingham [11], in reference to elastic liquids which do not follow Poiseuille's law, denies the property of viscosity to these substances and attributes plasticity to them. There are two objections to this viewpoint; first, the fact that elastic liquids (gelatin) deviate from Poiseuille's law is attributable to the presence of elastic qualities in the system [75, 88, 201]; and second, plasticity is already well understood to mean that property which permits deformation without any tendency to return to an original shape, which is not true of gelatin, protoplasm, or any elastic jelly. The difficulty in terminology might be escaped by using "consistency" to express the viscous (or plastic) qualities of elastic liquids. Bingham [11], Bogue [15], and others recognize a physical distinction in the type of (viscous) flow of water, glycerin, etc., and in the type of (plastic) flow of clay, gelatin, paint, etc. It is not our purpose to deny that such a distinction exists—though it does appear that the difference is asymptotic—but merely to point out that there is another explanation of the deviation of elastic liquids from Poiseuille's formula of viscous flow.

Weber [236, 237] has reviewed the subject of protoplasmic viscosity in detail, giving methods, data, and [239] an extensive bibliography.

ELASTICITY

The elastic properties of protoplasm are of interest chiefly because of the light which they throw on the structure of the living substance.

Attempts to measure the elasticity of protoplasm have been few. Many indirect observations have been made which clearly indicate that the living substance is highly elastic. Chodat [37] has called attention to the delicacy

and strength of the fine protoplasmic strands which connect a plasmolyzed protoplast to the walls of the cell. When these strands snap they contract markedly, indicating pronounced elastic qualities. The contractility of the chromatophore of *Spirogyra* was used by Scarth [191] as an indicator of protoplasmic elasticity and structure.

The tensile strength of protoplasm was determined by Pfeffer [179]. His experiments have been described.

No more convincing demonstration of protoplasmic elasticity is to be had than by stretching the living substance between microneedles. Two mechanically controlled glass needles are inserted into a plasmodium, an amoeba, an erythrocyte, or an isolated corpuscle nucleus, and separated. The protoplasm or cell can thus be stretched to a degree that varies greatly with different protoplasts and with the same protoplast at different times, i.e., at different physiological states. The protoplasm of myxomycete plasmodia is at times poorly elastic and at other times it may be stretched into very fine, long threads which return a goodly distance even though not fully to their original size. The amphibian erythrocyte varies in its elastic and plastic qualities. The red blood cell can usually be stretched to about three times its length [204]. Of extraordinary high stretching capacity is the isolated nucleus of an amphibian erythrocyte. A 14 μ nucleus can be stretched to a strand 350 μ long, and on release of the needles the nucleoplasm returns to nearly its original dimensions.

It is possible to determine the elastic value of protoplasm by inserting minute (16 μ) nickel particles into the living substance (of echinoderm eggs and plasmodia), and attracting these particles electromagnetically. On release of the current the metal particles return to their original position. The distance traveled by the particle in the living substance is measured with an ocular micrometer and is an indicator of the elasticity of the protoplasm. Values obtained are only crude measurements in the main, but bear comparison with each other and with non-living systems. A minimum value for the maximum stretching capacity is 4.4 μ for liquid, previously streaming protoplasm of myxomycetes; the maximum value is 292 μ for quiescent highly viscous exuded masses of protoplasm from plasmodia. The latter value is greater than any obtained by Freundlich and Seifriz [76] for gelatin solutions and slightly surpasses the maximum stretching value of fresh egg albumin. The technique was developed by Freundlich and Seifriz for the measurement of the elasticity of thin colloidal solutions.

Quantitative data on the elasticity of protoplasm are few and approximate only. Qualitative data are abundant, and point clearly to the conclusion that protoplasm is at all times elastic, varying only in degree, from the low stretching capacity of thin protoplasm through the high elastic limit of the soft protoplasmic jelly, to the resiliency of the firm protoplasmic membrane.

Very marked is the usual and almost complete loss of elasticity at death [204]. This can be interpreted as involving a general collapse in protoplasmic structure, or a complete reorientation of the structural units.

STRUCTURE

Any statement on the ultimate structure of protoplasm is going to be in the nature of a speculation, but there are sufficient data on the physical properties of living matter to make such speculations sound. A little is known

of the structure of some of the organic substances which constitute protoplasm. It is natural to suppose that the structure of the living heterogeneous system, while infinitely more complex, is of the same general fundamental character as is the structure of its component parts.

Protoplasm is an association of water, salts, carbohydrates, fats and proteins. The structure of protoplasm *in toto* is the structure of such an intimate mixture. Three essentially different types of systems are represented here, molecular dispersions (including ions), liquid suspensions, and jellies.

Whether we look upon the intimate association just described as representing, in its entirety, living matter, or whether we believe that some protein complex is the ultimate living substance, with salt and organic solutions comprising the internal environment, will not influence the result of our search for that structure which is the seat of certain physical properties of protoplasm.

Four physical properties of the living substance have been investigated with sufficient thoroughness to warrant using them as indicators of protoplasmic structure; these are imbibition, coagulation, viscosity, and elasticity. Of these four properties only viscosity is possessed by emulsions. Emulsions, in themselves, are not elastic (i.e., cannot be stretched),* do not coagulate (in the strict sense), and they do not swell. Proteins, on the other hand, possess all of these properties and they do so in virtue of a structural framework the units of which are linear in form.

Seifriz [203] has repeatedly emphasized that the seat of those processes which we characterize as vital is not to be sought for in an emulsion. A living emulsion is an impossible system. There are many reasons for believing this, some of which will later be mentioned. Here, for the moment, only one physical property, as a criterion of structure, will be considered, a property which does not distinguish living from non-living matter, but which is an ever-present property of protoplasm, namely, elasticity.

The statement that emulsions are not elastic, is, with some justification, often questioned. A pure oil and water dispersion is not, cannot be, elastic; but all commercial and practically all natural emulsions contain a stabilizing agent. It is the stabilizer which gives to an emulsion its elastic properties when it possesses such. The most successful emulsifiers are soaps, gums, albumins, and the like. It is the presence of these elastic substances in the dispersion medium, and especially in the stabilization membrane of concentrated emulsions, which give to these systems any elastic qualities they may possess. The case of rubber is an instructive one.

Freundlich and Hauser [74] have made a micro-anatomical study of the globules of the latex of Hevea. They find the outer coating of these globules of rubber latex to be an adsorption layer of soluble protein compounds which serve as emulsifiers. The remainder of the latex globule (its content) is a mixture of hydrocarbon with fatty acids and other minor rubber constituents. On coagulation of the latex the globules come into contact and adhere. The protein membranes which hold the globules of the coagulum in mutual contact give to rubber its elasticity [112].

It is customary in rubber manufacture to mill the crude product. Milling causes a breaking down of many globules and a consequent release of the enclosed hydrocarbon oil. Decreased elasticity results from increased milling. The explanation is evident. In unmilled rubber the oily content of the

* This is not invariably so, especially in complex emulsions; *vide infra*. J. A.

globules has no effect on the system since the hydrocarbon is fully sur-
rounded by the elastic protein jelly. With increased milling there is an in-
crease in released hydrocarbon oil, and the greater the milling the less is the
elasticity of the system.

An emulsion possesses elasticity only in virtue of the presence of an elastic
membrane of protein (or soap or the like).

The notion that colloidal jellies are fine emulsions was the basis of the
classification of colloids into "suspensoids" and "emulsoids." This termi-
nology and the concept on which it is based have been given up [70], and the
liquid suspensions put where they belong, namely, with the solid suspensions
[51, 53, 246]. Whatever the structure of the lyophiles may be, their physical
properties are quite distinct from those of emulsions. Emulsions and jellies
are as diverse as jellies and metal sols.

Hatschek [89], in an ingenious and mathematical analysis, has shown
that the theory that gels consist of two liquid phases must be pronounced
untenable. (See 3a, paper by Buchner.) Duclaux [51] believes that emul-
sions have little in common with the "true colloids." * Procter [182] has
made it appear very likely that the structural units of gelatin are fibers,
whether of molecular or colloidal dimensions is not clear. Bogue [15]
speaking of gelatin says that resiliency or elasticity is dependent upon the
length and number of catenary threads. Seifriz [201] has shown that the
microscopically visible structure of elastic soaps is fibrous in all cases, and
in certain instances crystalline, while the dispersed particles of inelastic soaps
are spherites. Leathes, whose chief contributions to science have had to do
with the rôle of fats in living systems [117], emphasizes the importance of
the protein constituents of protoplasm [118]. He points out that it is
the flexible cohesion of chains of amino acids which maintains the identity
of the protoplast. Wilson [242] looks upon a plate of gelatin as a continuous
network of chains of amino acids, there being no individual molecules unless
one wishes to regard the entire plate of gelatin as one huge molecule. A
hot solution of gelatin is a true solution consisting of individual gelatin
molecules.

That the colloidal particles of elastic sols are non-spherical is shown by
other physical properties than elasticity. Thus, Freundlich [72] calls atten-
tion to the double refraction of the vanadium pentoxide sol when subjected
to a magnetic field (Majorana phenomenon). This sol when stirred exhibits
streaks due to dityndallism or double diffraction. Zocher [245] has observed
long oriented particles in concentrated sols of benzopurpurin 4B and chryso-
phenin when hot solutions are cooled. In a magnetic field the sols become
anisotropic and reflect polarized light. (The reader's attention is called to
a valuable summary by Clayton [39] of colloid chemical research much of
which has important bearing on physiological problems.)

The whole trend of thought among physical chemists is definitely toward

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* It must here be borne in mind that colloidal particles may be of any shape, and even may be
tiny crystals—*size*, and not arrangement in a space lattice, is the criterion of colloidality. Single
molecules may themselves be large enough to be colloidal, in which case the "true" solution of the
substance may be colloidal. Furthermore, the concentration of the solution and temperature are factors
affecting the *time* required for particles (molecules or larger groups) to move into the positions of
minimum potential, towards which they strive. The tendency to form threads or other structures
may be opposed, delayed, or even reversed, before it attains its objective. Adhesion and cohesion,
without the formation of any definite chemical bond, can account for structures that persist under many
conditions, as a stamp sticks to an envelope. See [3].

As Edgar T. Wherry pointed out, particles may be colloidal only in one or two dimensions. For
a description of fibrous crystals of alkali halides, see J. E. Driver, *J. Soc. Chem. Ind.*, **46**, 197 T (1927).
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the conception of a linear structural unit for elastic jellies. The chains of amino acids, if the unit is of molecular dimensions, or the tenuous often crystalline fibers, if colloidal units build up the framework, form an entanglement, a "brush heap" to which the elastic properties of jellies are due. If we have to do with chains of amino acids, as Wilson [241] believes to be true, the cross section of any one thread would have the diameter of a single atom. That the structural units may be of colloidal size, or even, as in the case of soap curds [201], assume microscopical dimensions, is proven in some cases and seems very probable in others.

In connection with the possible molecular size of the structural units of jellies, it is interesting to note that Lumière [138] distinguishes two colloidal states of matter, the molecular and the micellary. It is quite clear that such an entanglement, a "brush heap" of interlacing crystalline fibers or amino acid chains, is elastic, while an aggregation of spherical structural units, as in emulsions, is inelastic. Once aware of these facts, and the further one, that protoplasm is highly elastic, the conclusion as to the primary structure of protoplasm is obvious.

That such a fibrous structure of living matter is in harmony with other facts is evident when one thinks of the wide prevalence of fibrous tissue in organisms. Nerves and muscles are bundles of fibers. Plants have fibrous tissues which are the foundation of certain commercial products in which tensile strength is an important factor. Individual cells have fibrous structures such as the neuromotor (anal cirri) fibers of the protozoan Euplotes [216].

Ettisch and Szegvari [56] find, with dark-field illumination, that the fibrous strands of frog connective tissue are built up of rod shaped micellae.

Bathing, permeating, this interlacing entanglement of fibers, is an emulsion of fats and an aqueous solution of salts, and other organic compounds in solution. Such a picture of living matter satisfies certain conditions as we now know them.

We cannot exclude the carbohydrates as possible factors in such physical properties of protoplasm as imbibition and elasticity. Cellulose is elastic and swells in water. Alexander [3] calls attention to the fact that concentrated solutions of lactose crystallize with difficulty when they assume an isocolloidal state. They then behave somewhat like a "glue." The proportion of cellulose in protoplasm is very low in plant cells and nil in animal cells, while the sugars are much below the proteins in quantity and apparently do not exist in a pseudocolloidal state. The chemical make-up of protoplasm (of myxomycetes) has been determined by Reinke [187] and by Lepeschkin [119] to be the following: proteins 55 to 62, fats 12, cholesterin 2 (Lepeschkin gives, lipoids 19), carbohydrates 12, salts 7, and other matter 5 to 12 per cent.

The concept of an interlacing mass of amino acid chains or slender crystalline fibers makes it possible to interpret the mechanism of some of the physiological properties of protoplasm, which cannot be done, or at least has not been done, on the basis of any other type of system. One such property is the immiscibility of protoplasm in water.

There has long waged a controversy over the miscibility or immiscibility of protoplasm in water [199]. The contention of those who support the miscibility side of the controversy, is that the living substance is kept from mixing in its surrounding aqueous medium—which it very evidently does not do—by the presence of the outer layer. That protoplasm when cut or

torn with microneedles immediately forms a new surface over the wounded part, is certainly true, but it is not this surface alone which prevents loss of the inner mass by diffusion in the aqueous medium. The protoplasm itself resists disintegration because of its own structure. An interesting example of this is the frequently to be observed struggle on the part of isolated bits of protoplasm to maintain their identity. A droplet of isolated protoplasm may not completely round up, part of its surface remaining ragged and exposed. There is no indication of miscibility at the still exposed region until a complete break-down in the entire mass occurs when the protoplasm quickly diffuses into the water or coagulates. The sudden disintegration of a protoplast make take place with the rapidity of an explosion. Eggs of echinoderms and protozoa frequently literally blow up. The taking in of water by protoplasm is an imbibition process and is not to be confused with miscibility in the sense of solubility.

As regards the crystalline character of the fibrous units of elastic jellies it is of interest to recall that Scherrer [196], Herzog [98], Sponsler [213], and others have determined a crystalline nature for such organic tissues and substances as silk, ramie fiber, nerve, muscle, hair, gelatin, cellulose, and starch. The method used is that of von Laue and of Bragg [17] wherein the three dimensional atomic lattice of crystal structure serves as a diffraction grating for very short waves of Röntgen rays. Diffraction, due to interference of X-rays at a number of centers in the space lattice formed by the atoms of which a crystal is built, produces a symmetrical pattern on a photographic plate. Organic tissues investigated, silk for example, show remarkably clear diffraction diagrams, certain evidence of a crystalline structure.

An interesting problem presents itself in connection with the fluid and the elastic properties of liquid jellies. A soap whose stretching capacity can be demonstrated may yet have only twice the consistency of water [76]. How, then, can we account for such marked fluid and rigid (elastic) qualities in the same system? If we imagine the lineal structural units loosely adherent at their tips, there will be sufficient "rigidity" to account for the elastic properties, and yet the structural units will be joined loosely enough to permit smooth flowing. Szegvari [215] advances a similar hypothesis. In a jelly of higher viscosity and greater elasticity the fibers are more closely interlocked. In the more liquid state of protoplasm, then, the structural units of the framework, the amino acid chains of the protein-complex, intertwine but little and touch only at the ends, bound loosely by mutual adsorption. Ease in flowing is accountable for by the dragging of the linear units at their points of contact which in the dilute liquid state is at the ends only. The more interlocked the fibers become the less readily does the protoplasm flow and the higher is the elasticity.

A striking example of the sudden change in protoplasmic structure— involving in all probability, a quick passing from a close and firm interlocking of the linear structural units to a more loosely bound framework—is to be seen in the instantaneous collapse of the mitotic figure of a dividing echinoderm egg. If an egg in mid-mitosis is subjected to the tension of the surface film of a thin layer of water on a cover-glass, the mechanical pressure causes a complete break-down of the mitotic figure leaving no vestige of the previously existing astral rays, spheres, and spindle. The collapse is so sudden and so complete that it gives every evidence of being purely structural. This rapid change in cell configuration, due simply to mechanical pressure, is simi-

lar to the sudden liquefaction of an iron oxide gel described by Schalek and Szegvari [195] and to a cadmium gel described by Svedberg [214]. In both cases the firm gel is reduced to a thin sol simply by mechanical disturbance (shaking or stirring). The cadmium solution (metallic cadmium in alcohol) is of but 0.2 per cent concentration, yet the gel formed is very firm and is, by stirring, reduced to a sol scarcely more viscous than alcohol.

Let us carry this picture of protoplasmic structure to the surface where lies the mechanism of permeability control. Here we have essentially the same type of system; a fibrous entanglement bathed by aqueous solutions and fatty emulsions. The only marked physical distinction between the morphological membrane and the interior protoplasm is one of degree of rigidity. The surface film is a firmer and more compact layer, a closer interlocking of the linear units. But this condition is not fixed. The protoplasmic membrane is just as capable of changes in consistency as is the endoplasm. It is a fundamental fallacy to regard the morphological protoplasmic membrane as an essentially different type of system from the interior of the protoplast.

To attempt to give any precise picture of the actual configuration of the protein framework of living matter, is quite impossible. Leathes graphically describes the tremendous complexity of the problem of the structure of proteins.

Leathes [118] assumes "a very simple case" of a protein built up of only fifty amino acid links. If instead of all being different, one link recurred ten times, four recurred four times, and ten recurred twice, then the number of possible permutations would be $\dfrac{\lfloor 50}{\lfloor 10} \times (\lfloor 4)^4 \times (\lfloor 2)^{10}$. Such a protein would consist of a chain of fifty links of which only nineteen are different. The number of possible arrangements of its parts would be 10^{48}. Light takes 300,000 years to travel the length of the Milky Way. This distance expressed in Ångstrom units, of which 10,000,000 equal a centimeter, would be less than 10^{32}. It is thus clear how great the variations in disposition of the parts of a protein molecule may be, and how far we are from being able to map out such a structure.

While emphasis is laid upon the proteins as the basic material of living matter, attention should be called to the nucleic acids, so abundant in nuclei, and one of the chief constituents of those important heredity-carrying structures, the chromosomes. There can be no doubt but that the nucleic acids are fundamental to life. They are non-protein in character but there is little reason to believe that they exist as such in protoplasm. The nucleic acids are firmly bound with the protein part of the nucleoproteins. They may exist as protein salts but more likely are in closer combination with their protein associates, as is the case in haemoglobin, which is a firm union of the non-protein haematin (and iron) with the simple protein (histone) globin. It seems impossible to dissociate the nucleic acids from their protein associates. Similar bonds between the non-protein and the protein constituents of living matter are known; thus, the sugars unite to form the glucoproteins and the fats to form the lipoproteins. The intimate association of the nucleic acids with the proteins of chromosomes, which play so important a part in life as determiners of what we are and what we do, adds, rather than detracts, from the fundamental rôle of the proteins in vital processes.

The problem of the structure of protoplasm and of non-living jellies is

an extraordinarily difficult one. Some few facts stand out as apparently well established. The structural unit of elastic proteins and of the protoplasmic framework is linear. The unit may consist of chains of amino acids and therefore be of atomic dimensions, or the amino acid chains may, in certain cases, aggregate and form units of colloidal proportions, which may be crystalline in nature.

While denying to fats and emulsions any prime part in the more fundamental properties of protoplasm, and in particular to those physical properties which we have been considering, we do not thereby rob them of the very evident secondary rôle which they play in vital phenomena, namely, principally, as nutrient matter.

The identity of a protoplast, in other words, life itself, is made possible by continuity in the structure of protoplasm; and this continuity is that of an elastic framework of a protein complex.

CONCLUSION

Few theories in the world of science have held their own for more than a decade. It may, perhaps, be too much to hope that the theories here presented on the physical properties of protoplasm will prove an exception. We can only trust that we are working in the right direction. In realizing that nothing has been fully settled, we are happy in the thought that a few undeniable facts stand out as the contribution of present-day physical biology to our knowledge of the constitution of protoplasm.

Studies on the nature of living matter form the basis of all knowledge of the dynamics of vital phenomena. Only through a thorough understanding of the physical properties of protoplasm can we learn more of protoplasmic behavior. The problems of the behavior of organisms, of growth, reproduction, heredity, and disease, are the problems of the colloidal physics of protoplasm.

It is a pleasure for the writer to express his appreciation of the valuable suggestions which he, during the writing of this article, has received from his friends and colleagues in biology, physics, and chemistry. To Professor Howard E. Pulling especial thanks are due for his careful reading and helpful criticism of the manuscript of this article. The subject here discussed is a very big one, much of which we still know little of. Only the sympathetic constructive cooperation of all will ultimately lead to some understanding of the many difficult problems involved. Such cooperation has made this article possible. While the writer has laid emphasis on the point of view which he favors, he trusts that he has in all instances given fair consideration to the opposite viewpoint.

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Protoplasm

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Nineteenth century biology demonstrated the essential similarity of all living things. The most diverse types of animals and plants all contain a material known as protoplasm. This material, though never quite the same in any two plants or animals or in any two parts of the same animal, is nevertheless surprisingly uniform in its properties and its behavior.

Seen through the microscope it commonly appears hyaline, with granules scattered through the entire mass. It occurs in the form of cells, microscopic globules of protoplasm which retain their independent identity. Generally each cell has within it a tinier globule which is sharply distinct from the rest of the cell and which constitutes the nucleus.

Not only do all types of protoplasm have a general form of resemblance but they resemble each other chemically. When plants and animals are analyzed, it is found that in all instances they are composed of the same types of chemical compounds. Thus the protoplasm concept, which was first established on the basis of form alone, was strengthened by the later chemical knowledge which showed a general underlying similarity in the chemical materials of life.

Protoplasm is largely water. Our bodies are two-thirds water, and if we exclude the harder structures like bone and teeth, the organs richer in protoplasm contain three-fourths to four-fifths water. They are just about as watery as the blood. Some animals and plants contain even higher percentages of water. In addition to water the other inorganic constituents contained in protoplasm are salts. The commonest salts are the chlorides of sodium, potassium, calcium, and magnesium, but other salts are also found. Physiological study has shown many times that the salts are a very essential constituent of protoplasm.

The organic constituents of living matter are the proteins, the fats and lipoids, and the carbohydrates. Concerning the various types of proteins and other organic constituents found in protoplasm a huge body of knowledge has been built up and this knowledge is readily accessible in the many books which discuss physiological chemistry. Only one point needs emphasis here. Protoplasm in every case includes two types of materials, those soluble in water and those thoroughly insoluble. Most proteins dissolve in water, but fats and lipoids are characterized by their total insolubility. As might be expected the fats and lipoids of protoplasm are present in the form of an emulsion or suspension.

It should be pointed out that our chemical knowledge of protoplasm is far from complete. The biochemist is under a serious handicap, for as soon as he begins his manipulations the protoplasm dies and this death always involves chemical changes. It is not even certain that protoplasm is a mixture

of chemical substances rather than a single chemical compound. Such prominent physiologists as Pflüger, Ehrlich, and Verworn have held that protoplasm is a compound of huge molecular size. However, this view does not appear very plausible at the present time.

Whatever the chemical make-up of protoplasm may be, there is universal agreement that it is a colloidal material in the ordinary sense of the term. There can be no question but that it is a very complex colloidal material. In it water is doubtless the dispersion medium.* Living cells behave as tiny osmometers. When they are placed in solutions above a definite molecular concentration water passes out of the cells, and water enters the cells when they are placed in more dilute solutions. This indicates that the interior of the cells contains water in a free form, in other words that water is the dispersion medium. The fatty materials of the cell are scattered through it in the form of an emulsion or suspension, as can in most instances be demonstrated by microscopical study. In addition to the fatty particles there are many other granules in protoplasm which are not fatty. These are usually composed of protein material. Protoplasm contains not only visible particles, it also contains particles too small to be seen with the ordinary powers of the microscope. These can be demonstrated with darkfield illumination, and they are doubtless colloidal particles in the ordinary sense. Thus protoplasm is a colloidal solution in the strict sense as well as an emulsion or suspension. And finally since the water which forms the dispersion medium contains dissolved salts, it is also to some extent a true solution. Protoplasm is complex, but such a triple complexity is not at all unusual. Thus milk is at one and the same time, a true solution, colloidal solution, and an emulsion, and many of the ordinary emulsions of commerce really contain all types of dispersion.

Physiologists are generally agreed that the essential properties of protoplasm are due in large measure to its colloidal nature. In physiological theory it has been the custom to postulate colloidal changes in the attempt to explain life processes. Such theories have in practically all cases been based on pure speculation. Most theories of muscular contraction, for instance, postulate a colloidal swelling of the muscle proteins, but there is no direct evidence in favor of such a view, and it is just as plausible to assume a coagulation. In order to solve the riddle of colloidal change in protoplasm, some authors have brought forward experiments on gelatin and other colloids. This method of reasoning is not of very great value. Proteins differ very widely among themselves in their colloidal behavior and protoplasm is certainly like no one of them.

Any study of the colloid chemistry of protoplasm would of necessity be difficult. It is always hard to study the colloidal behavior of substances whose chemical nature and reactions are unknown. The colloidal study of proteins is an extremely complex problem in itself. But in the case of protoplasm the situation is even worse. Protoplasm is apparently a mixture of chemical substances, most of which are of unknown composition. It is a mixture in which the proportions can in no way be controlled, for the investigator has not made the mixture and he can not materially change it. Perhaps the worst difficulty in the study of protoplasm is the fact that it is more or less inaccessible, or at any rate it can not be manipulated in the ordinary ways. One

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* See paper by Martin H. Fisher in this volume; he regards the water as being dispersed in the protoplasm. *J. A.*

can not pour a few cubic centimeters of protoplasm into a test-tube and study it there. Living substance when it is alive is always found in the form of cells, and these cells are minute, on the average about a hundredth of a millimeter in diameter. Small wonder then that progress has been slow, that biologists until recently did not know whether protoplasm was a fluid or a jelly.

Fortunately in recent years it has become possible to decide when gelation or liquefaction of protoplasm occurs. This is due primarily to the discovery of methods of measuring the viscosity of protoplasm within the cell.

The first method of determining protoplasmic viscosity is due to the German botanist Heilbronn.[1] It is of course not possible to determine protoplasmic viscosity by observing the flow of the living substance through a tube. Heilbronn measured the rate of fall of starch grains through protoplasm and compared it with the rate of fall of the same grains in distilled water. His method is quite sound from a physical standpoint, although he makes no correction for the retarding effect of the wall of the cell. Such a correction would probably be only a slight one, and Heilbronn's results are very valuable. Unfortunately his method is not applicable to animal cells, for although these cells have many granules, no movement ordinarily occurs under the influence of gravity. A stronger pull is necessary.

In 1915, Heilbrunn[2] working on sea-urchin eggs was able to determine the relative viscosity of this type of protoplasm at different times and under different conditions. Heilbrunn's measurements do not give the absolute viscosity of the protoplasm and for this reason they are not as valuable as Heilbronn's, but they were made on active cells and they give information concerning viscosity changes in living protoplasm. Thus Heilbrunn showed conclusively that biological activity, in one case at least, is associated with colloidal changes. Heilbrunn's methods are described in various papers[3] and only a brief reference to them is necessary here. When marine eggs are centrifuged the granules separate into zones (*see* Fig. 1). The number of seconds necessary for such a movement of granules is taken as a measure of the viscosity.

Following Heilbrunn other workers[4] were able to detect colloidal changes in cells by "microdissection," that is to say, by the dissection of cells under the microscope with the aid of needles manipulated mechanically. These methods have an advantage in that they may give information concerning local regions of the cytoplasm. On the other hand, they are not very accurate, and it is rather obvious they tend to be too subjective.[5] Recently Heilbrunn[6] has described a new method of viscosity measurement which depends on the movement within a cell of a bit of metal drawn by a magnet. Darkfield illumination has also been used to detect colloidal changes in cells.[7]

The physical study of protoplasm has so far progressed only a short distance. But some facts have been discovered, and it is certain that the way is now open for further progress.

Protoplasm is fluid. It is not a jelly as many have supposed. Heilbronn

[1] *Jahresber. wiss. Bot.*, **54**, 357 (1914).
[2] *Biol. Bull.*, **29**, 149 (1915).
[3] *J. Exp. Zool.*, **30**, 211 (1920); *ibid.*, **34**, 417 (1921).
[4] Chambers, *J. Exp. Zool.*, **23**, 483 (1917); *J. Gen. Physiol.*, **2**, 49 (1919). Seifriz, *Bot. Gaz.*, **70**, 360 (1920).
[5] Heilbrunn, *J. Exp. Zool.*, **34**, 417 (1921); Heilbronn, *Jahresber. wissensch. Bot.*, **61**, 284 (1922). [See, however, paper by Robt. Chambers in this volume. *J. A.*]
[6] *Loc. cit.*
[7] Leblond, *Compt. rend. Soc. Biol.*, **82**, 1150, 1220 (1919); Bayliss, *Proc. Roy. Soc.*, ser. B, **91**, 196 (1920).

found both in higher plants [8] and in slime moulds [9] that the viscosity of the protoplasm varies from about ten to twenty-five times that of water. This is not a very high value, for many liquids have a much greater viscosity. Thus the relative or specific viscosity of olive oil (compared to water) is about 90, and the specific viscosity of glycerin is around 800. Heilbronn's measurements show that the protoplasm within the cells examined by him is no more viscous than ordinary sulfuric acid. Doubtless the viscosity of protoplasm varies in different stages of activity. Thus Leblond [10] found that during quiescent periods the protopalsm of various algae showed much less Brownian movement and was probably much more viscous than during periods of activity. There are also rapid changes in viscosity during cell activity as will be shown later. It should also be noted that the outermost layer of protoplasm is much more rigid than the interior.[11]
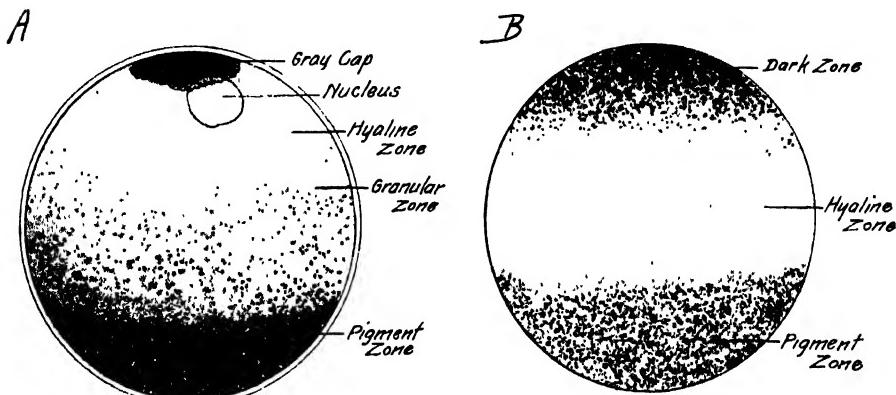
Fig. 1.

A.—Centrifuged egg of the sea-urchin Arbacia, after Lyon and Morgan.
B.—Centrifuged egg of the clam Cumingia, after Morgan.

In a colloidal study of protoplasm it is interesting to determine how the viscosity changes with the temperature. Some colloids change from gel to sol as the temperature is raised, others become coagulated. The behavior of protoplasm is complex, but it is surprisingly similar in the widely different types of cells which have been studied. F. and G. Weber [12] showed that in the cells of bean plants (Phaseolus), a rise in temperature caused a slight decrease in viscosity. Heilbrunn [13] showed that lower temperatures also produce a decrease in viscosity in the protoplasm of sea-urchin eggs, and this result was confirmed for slime molds by Heilbronn.[14] Weber and Hoheneg-ger [15] have found that temperatures near the freezing point cause an increased viscosity in the protoplasm of bean cells. In a recent paper Heilbrunn [16] has plotted the temperature viscosity curve for the protoplasm of the eggs of the

[8] *Jahresber. wiss. Bot.,* 54, 357 (1914).
[9] *Jahresber. wiss. Bot.,* 61, 284 (1922).
[10] *Loc. cit.*
[11] Heilbrunn, *Biol. Bull.,* 29, 149 (1915).
[12] F. and G. Weber, *Ber. deut. botan. Ges.,* 34, 836 (1917).
[13] Heilbrunn, *Biol. Bull.,* 39, 307 (1920).
[14] Heilbronn, *Jahresber. wiss. Bot.,* 61, 284 (1922).
[15] Weber and Hohenegger, *Ber. deut. Ges.,* 41, 198 (1923).
[16] Heilbrunn, *Am. J. Physiol.,* 1927.

clam Cumingia (Figs. 2 and 2a). This curve is not very accurate, as it is not yet possible to measure protoplasmic viscosity with anything like the accuracy possible in the measurement of the viscosity of ordinary liquids. However, the viscosity differences are so striking that the main course of the curve is doubtless correct. In the clam eggs, the protoplasmic viscosity passes through a maximum at 15 degrees, and a similar maximum doubtless occurs in the protoplasm of slime molds (Heilbronn). At temperatures either above or below 15 degrees the viscosity drops until it undergoes a sharp increase both at low and at high temperatures. This viscosity increase is apparently due to a gelation or a coagulation of some constituent or constituents of the protoplasm. It seems probable that the temperature curve of protoplasmic viscosity will eventually be related to many details of plant and animal life.

In recent years colloid chemists have devoted a large share of their attention to the electric charges of colloids. Some colloids bear a positive charge, others a negative charge. The source of these charges is at present the subject of much discussion, but this discussion need not concern us here. The biologist is interested in knowing whether the colloids of living cells are positive or negative, and such knowledge is essential to any understanding of protoplasmic dynamics.

With ordinary colloids the usual method of deciding whether the colloidal particles are charged positively or negatively is to observe their migration in an electric field. This method is not very easy to apply to living cells for there is good evidence that the colloids on the inside and the outside of living cells are charged oppositely. Probably the migration of cells in an electric field is determined largely by the nature of the surface charge alone.

Since it is not an easy matter to test the migration of protoplasmic particles, another method of determining the electric charges of protoplasm was used.[17] The principles governing this method are discussed in various books on colloid chemistry,[18] and only a brief reference is possible here. It is commonly held that positively charged colloids are more easily precipitated by salts with readily adsorbed anions, and negatively charged colloids are more easily precipitated by salts with readily adsorbed cations. Generally speaking, bivalent ions are adsorbed to a much greater extent than monovalent ions. In the presence of calcium and magnesium salts there would be a greater adsorption of positively charged ions than in the presence of sodium, potassium, or ammonium salts. Hence a given concentration of calcium and magnesium ions would be more effective than a similar concentration of monovalent ions in causing a coagulation of negatively charged colloids, but it would be less effective in the coagulation of positively charged colloids. Normally protoplasm contains and is surrounded by both these types of salts. When the percentage of calcium or magnesium salt is increased, then the amount of positive charge adsorbed on the protoplasmic particles should become larger. This would tend to precipitation if the protoplasmic particles were normally charged negatively, but it would have an opposite effect if the particles of protoplasm normally bore a positive charge. Thus by increasing or decreasing the proportion of calcium salts and observing the effect on the protoplasm one can decide the question as to the nature of the charge on the protoplasmic particles.

The results obtained with this method are decisive. Both in sea-urchin

[17] Heilbrunn, *Am. J. Physiol.*, 64, 481 (1923).
[18] Bancroft, "Applied Colloid Chemistry," New York, 1921, p. 211.
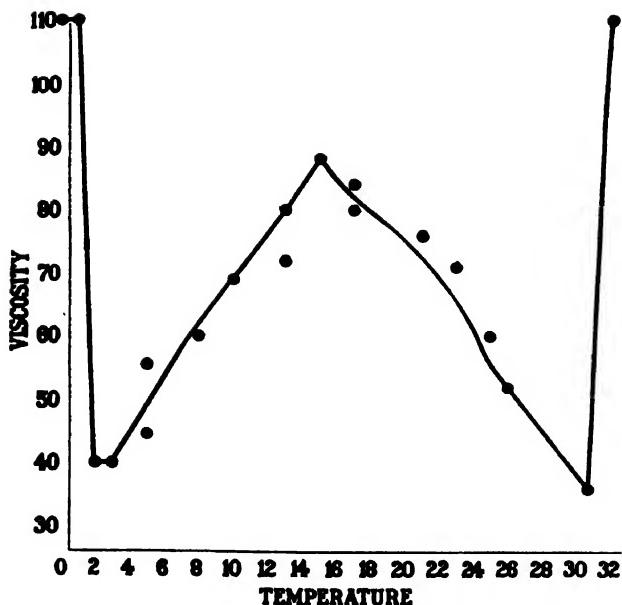
FIG. 2.—The viscosity of Cumingia egg protoplasm at various temperatures.
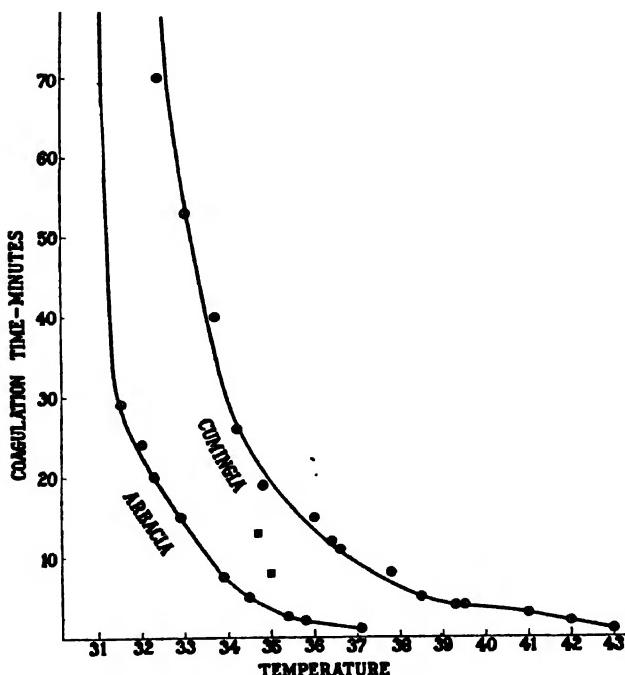
FIG. 2-A.--The time of heat coagulation of Cumingia egg protoplasm at various temperatures.

eggs and in the protozoön Stentor, an increase in the proportion of calcium and magnesium ions, instead of exerting a pronounced coagulative action, tends to liquefy the protoplasm within the cells, whereas an increase in the proportion of sodium, potassium, and ammonium ions coagulates the protoplasm.[19] The results are clear-cut, and they are not due to any influence of the hydrogen-ion concentration which was kept approximately constant. The evidence is strong, therefore, that the interior mass of the protoplasm is positively charged. On the other hand a thin outer layer of the cell bears a negative charge, for it tends to become more fluid in sodium salts as compared to calcium salts.

From these facts it appears that living cells have positive charges within and negative charges at their surface. In support of this view various older observations can be cited. It is well known that bacterial cells migrate to the anode when placed between two electrodes.[20] In small cells of this sort the surface charges play an all-important part, and the negative charge on the cell exterior would carry it to the anode. Larger cells behave somewhat differently. Lillie[21] found that when the white blood cells of the frog are placed between two electrodes, the smaller cells migrate to the anode, but the larger cells go to the cathode. In the larger cells the interior mass is relatively greater in proportion to the surface and the relatively larger number of positively charged particles inside the cell would send it to the cathode. When an electric current is sent through the large cells of some protozoa, the protoplasm bulges towards the cathode.[22] All these facts favor the view that the interior of protoplasm contains positively charged colloidal particles, while the outer layer consists of negatively charged particles.

This new knowledge concerning the electric charges of protoplasm and the effects of sodium and calcium ions on protoplasmic viscosity affords a real explanation of one of the most puzzling problems of physiology. For many years it has been known that sodium and calcium ions have antagonistic effects on all sorts of living protoplasm. The reason for this antagonism has remained a mystery, although Höber[23] has suggested that the two ions have opposite effects on the colloids of the cell. Höber believed that sodium had a liquefying action and calcium a coagulating action. The experimental evidence shows the reverse to be true.

When cells are active, the fluidity of the protoplasm does not remain constant. During cell division sharp and rapid changes in protoplasmic viscosity occur. The best known case is that of cell division. In this instance it has been shown during the mitotic or division process, the protoplasm becomes first stiff, then fluid, then stiff again.[24] In the case of the Cumingia egg, it has been possible to plot a curve which shows the changes in viscosity during several divisions of the cell[25] (see Fig. 3). The details of this curve cannot be discussed here, but it is of interest to note that the viscosity of the protoplasm increases and decreases very suddenly. What actually happens is probably the coagulation or gelation of some material within the protoplasm, the coagulation presumably resulting in the formation of a structure known to biologists as the mitotic spindle. Indeed the mitotic spindle can be made to

[19] Heilbrunn, *loc. cit.*
[20] For references to literature on this subject see Putter, *Z. Immunitätsforsch.*, **32**, 538 (1921).
[21] *Am. J. Physiol.*, **8**, 273 (1903). [See also paper by R. S. Lillie in this volume. *J. A.*]
[22] See Jennings, "Behavior of the Lower Organisms," New York, 1906, p. 12 and p. 84.
[23] Höber, *Arch. gesammt. Physiol.*, **182**, 104 (1920).
[24] Heilbrunn, *J. Exp. Zool.*, **30**, 211 (1920).
[25] Heilbrunn, *J. Exp. Zool.*, **34**, 417 (1921).

appear and cells in general may be made to divide by treating them with chemicals of such a sort as to cause coagulation within the protoplasm.[20]

Apparently all sorts of vital activity are closely bound up with colloidal changes in the protoplasm. Living processes of the most diverse types are due to the same sort of changes in the protoplasm, for practically all manifestations of life may be reversibly inhibited by the use of certain anesthetics. For example ether in about the same concentration not only prevents consciousness in man, it prevents the movement of a fish, the wriggling of an earthworm, or the activity of a plant cell. Ether prevents protoplasmic activity
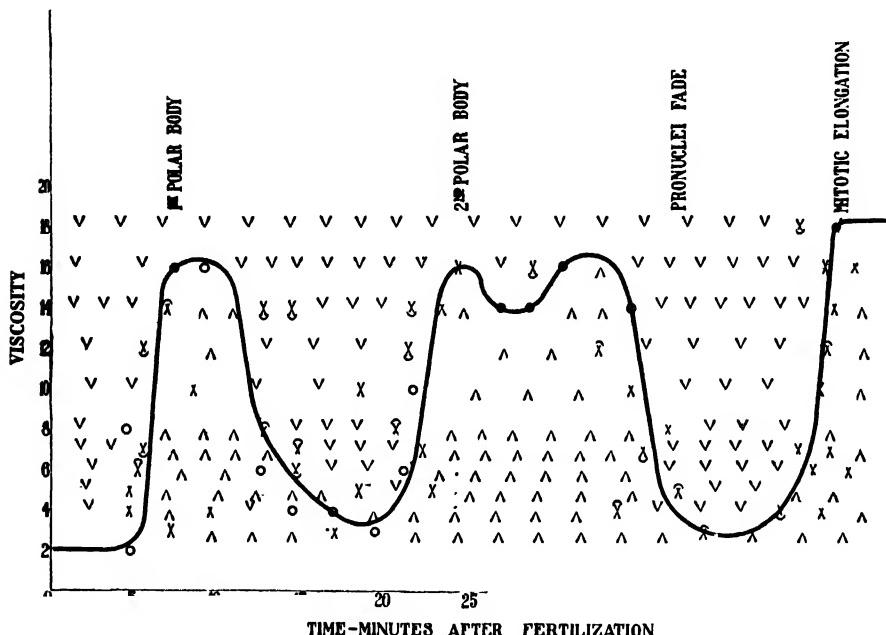
Fig. 3.—Viscosity changes during cell division in the egg of Cumingia. In this graph the viscosity values are relative, and the scale differs from that of Fig. 2, each unit here representing about 40 units of the scale of Fig. 2.

without killing the cell, and apparently its action is much the same in all instances. One of the most important questions that physiologists have attempted to answer is the nature of the effect produced by anesthetics. It has been shown that the division of the sea-urchin egg may be prevented either by keeping the protoplasm fluid or by keeping it in a relatively stiff condition.[27] Ether and most types of anesthetics keep this type of protoplasm fluid, although in concentrations above that which is best for anesthesia they cause coagulation. In plant protoplasm Heilbronn[28] showed some years ago that ether produces a gelation effect. Since then Weber[29] has found that dilute concentrations of ether make plant protoplasm more fluid, whereas slightly stronger

[20] Heilbrunn, *Biol. Bull.*, 29, 149 (1915).
[27] Heilbrunn, *Biol. Bull.*, 39, 307 (1920).
[28] Heilbronn, *Jahresber. wiss. Bot.*, 54, 357 (1914).
[29] Weber, *Biochem. Z.*, 126, 21 (1921).

solutions cause it to gel. There is good evidence that anesthesia is due primarily to an effect on the protoplasm of the cell of such a sort as to prevent sudden changes in colloidal condition. If this is true then it is these changes in colloidal condition that are the essential factors in life processes.

Of course only a beginning has been made in the colloidal study of protoplasm. More abundant data and more exact data are greatly needed. Many important questions have not been considered at all. It is of great interest to know what causes the changes in colloidal state which occur in living cells. So far there has been no real attempt to solve this problem, although Jacobs [30] has published some very suggestive experiments on the effect of carbon dioxide on protoplasmic viscosity. Carbon dioxide is almost universally produced during vital activity, and for this reason Jacobs' work is especially important.

[30] Jacobs, *Biol. Bull.*, **42**, 14 (1922).
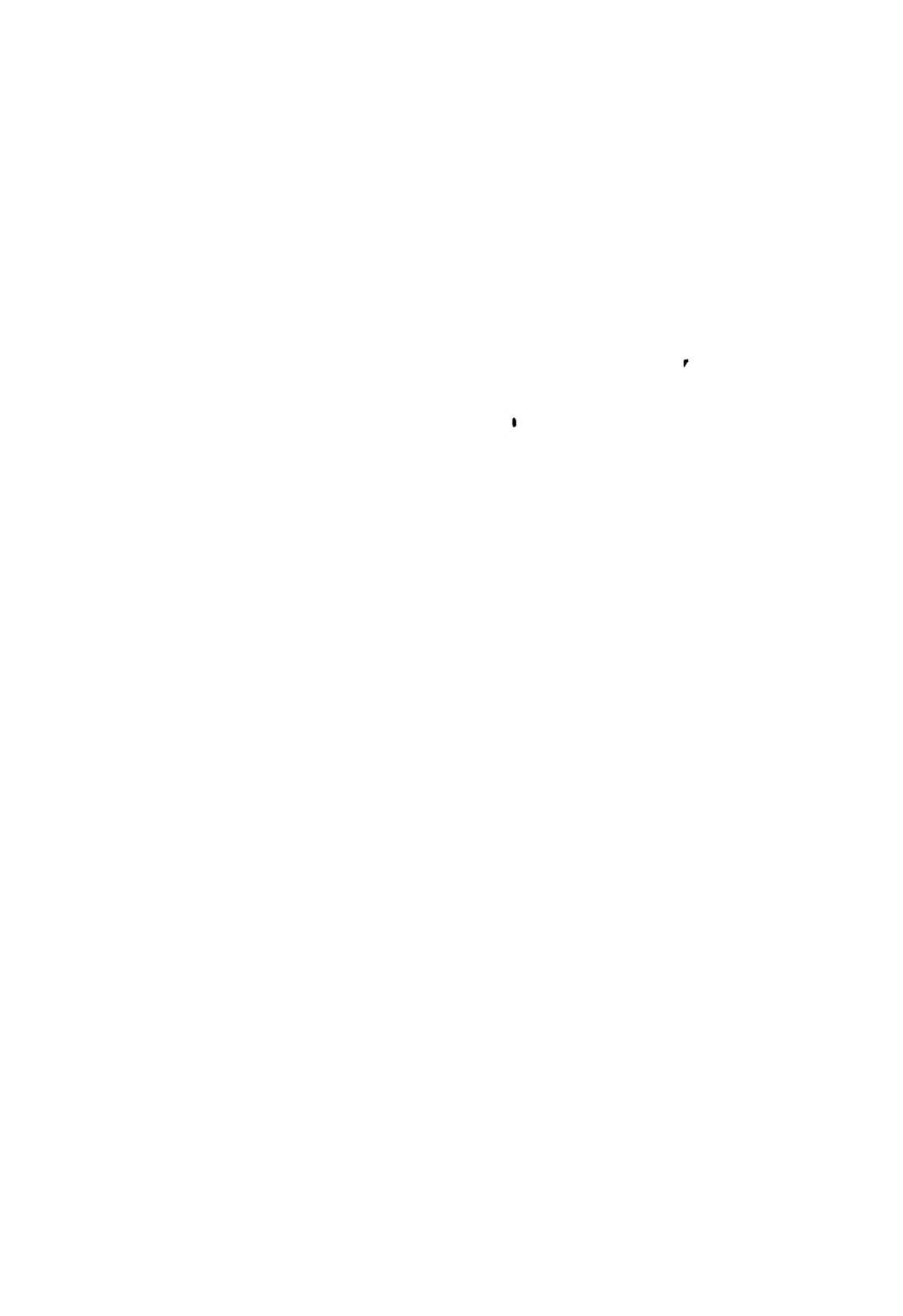
The Colloidal Structure of Protoplasm and Protoplasmic Action

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In a sense it is a truism that colloids form the basis of protoplasmic structure, yet the statement has a certain heuristic value, since it implies that the investigation of colloids may be expected to furnish the key to the understanding of this structure and of the special "vital" activities which are based upon it. The properties of living matter, however, are not to be understood in the basis of its colloidal constitution, considered alone and in a static sense. The fundamental vital characteristic is metabolism, i.e., continuous chemical activity, especially of a constructive kind. Protoplasmic structure is itself a product of metabolic activity and is maintained by that activity; once formed, this structure secondarily influences metabolism; but metabolism is the primary factor in all cases. The property of growth, implying the metabolic synthesis of specific proteins and of the other compounds which form the basis of protoplasmic structure, is inherent in all forms of living matter. The essential problem to be solved has reference to the special nature of the relations between the colloidal structure of living matter and those chemical activities which are chiefly characteristic of it.

Associated with and largely determining the syntheses underlying organic growth, are various energy-yielding reactions, chiefly oxidations, of which the oxidation of carbohydrate is the most important. These reactions furnish the energy exhibited in the movements and other activities of the organism; in the protoplasmic system they proceed rapidly at low temperatures ($<40°$); and one of their most remarkable features is that their velocity is subject to wide variation under the influence of the impinging forces of the environment. This feature constitutes the basis of the property known as "irritability," universal in living protoplasm. The structure-forming reactions, i.e., the syntheses underlying growth, are also subject to the modifying influence of external "stimuli." The determination of the conditions underlying this variability of reaction-velocities, with its often extreme sensitivity to changes of external conditions, is essential if we are ever to understand in any exact physical sense the properties of living matter.

The problem of the relations between protoplasmic structure and metabolism may be approached in various ways. The dependence of the basic metabolic reactions (oxidations, syntheses) on the preservation of a particular type of structure is readily shown experimentally. The consumption of oxygen and the production of CO_2 are typically greatly decreased when protoplasmic structure is destroyed, mechanically or by chemical (cytolytic) agents; at the same time the power of maintenance and growth (synthesis) disappears. Structurally disintegrated protoplasm is chemically inactive or "dead" protoplasm. Certain reactions are thus intimately dependent on the intact pro-

toplasmic structure. Many such reactions belong clearly in the class of heterogeneous catalyses. Warburg [1] has recently shown that oxygen-consumption in suspensions of disintegrated cells is associated with the solid (centrifuged) part of the residue; these fragments of solid protoplasmic structure influence certain oxidations in the same manner as the particles in a suspension of charcoal; e.g., soluble amino-acids may be oxidized under the influence of a suspension of animal charcoal as rapidly as in living protoplasm. These oxidations are depressed by cyanide and by surface-active organic compounds of the anesthetic group (alcohols, urethanes), and accelerated by small quantities of iron salts; such behavior is also highly characteristic of living protoplasm. Parallels of this kind indicate plainly that the chief protoplasmic reactions occur under the catalytic influence of surfaces; in other words, that the polyphasic structure of protoplasm is a primary factor in the determination of its peculiar type of chemical behavior.

This conclusion, while important, throws little light on the problem of the special or detailed nature of protoplasmic structure as distinguished from the structure of those simple heterogeneous systems like suspensions of charcoal or platinum which exhibit a similar strongly marked catalytic activity. Obviously protoplasm is a heterogeneous system of some kind; the physiological problem relates to its special features of structure, composition and arrangement of components. Its irritability and its remarkable power of synthesizing new and complex compounds must have as their counterparts structure of a highly definite kind, not present, or imperfectly developed, in colloidal systems of the usual inorganic types.[*]

It is not possible here to discuss the different biological conceptions of protoplasmic structure; but it may be pointed out that Bütschli's theory, according to which the basic structure is a foam-work or emulsion-like system, is the one most consonant with our modern conceptions, since it implies that the protoplasmic organization consists essentially of an arrangement of separate phases bounded by stabilizing interfacial films. We know from many features in the structure and behavior of protoplasm that the presence of continuous semi-permeable films or membranes, enclosing structurally distinct regions, is intimately bound up with its special type of activity. Protoplasm is a film-partitioned system. The presence of membranes enclosing small protoplasmic masses ("cells"), or surrounding the special structures or differentiated areas in the interior of cells (such as nuclei, vacuoles, fibrils, spheres), has long been regarded as a structural feature having fundamental physiological significance. Briefly, we may say that the cellular structure of living matter resembles emulsion structure chiefly in the importance of the part played by interfacial films.

The conception of protoplasm as a film-bounded and film-partitioned system throws light on many of its hitherto unexplained peculiarities, in particular its characteristic property of irritability. Irritability imples responsiveness to the electric current. Electrical sensitivity, as Hermann inferred and Nernst first demonstrated, is a function of polarizability. Lapicque, Lucas, and others have shown that the essential condition of electrical stimulation in irritable tissues is the production of a certain degree of electrical polarization within a certain time.[2] Polarizability signifies resistance to the passage

[1] *Biochem. Z.,* 119, 134 (1921).
[*] See paper on Inorganic Ferments, by G. Bredig, this volume. *J. A.*
[2] For references to the literature in this field see my recent book, "Protoplasmic Action and Nervous Action," University of Chicago Press, 1923, Chapter 12.

of ions; and all the evidence indicates that the chief seat of this resistance is the semi-permeable membranes of the irritable elements. Electrical sensitivity is therefore to be referred to the presence of polarizable or semi-permeable boundary-films or membranes enclosing or partitioning the protoplasmic system.

The conditions of permeability of the protoplasmic membranes (especially of the surface-films of cells or plasma-membranes) have recently been the subject of much investigation, but the intimate connection between semi-permeability and the electrical sensitivity of living matter has not hitherto been recognized with sufficient clearness. Protoplasm is electrically sensitive only so long as its film-structure is intact. On death semi-permeability and polarizability are lost, simultaneously with electrical sensitivity; electrical sensitivity also disappears temporarily in irritable tissues (muscle and nerve) during the brief period (known as the refractory period) immediately following stimulation, at which time there is evidence of a temporary loss of semi-permeability and decrease of electrical polarizability.

The fact that the irritable system responds to the electric current shows that the chemical reactions in protoplasm are directly influenced (initiated, accelerated or retarded, according to conditions) by the passage of the current. The significant fact from the physico-chemical point of view is that this influence is bound up with the polarizability of the protoplasmic films or membranes. Apparently when an electric current is passed through a living tissue (e.g., muscle) the resulting change of polarization at the membranes traversed by the current is the essential factor determining the initiation or release of the chemical reactions whose effect is seen in the succeeding response. The conditions are closely comparable with those at the surface of a metallic electrode when a current passes between it and the adjoining electrolyte-solution. In the latter case the attainment of a sufficient degree of polarization (shown in the existence of a certain critical decomposition-voltage) is followed by the initiation of chemical reactions (oxidations and reductions) at the surface of the electrode. Many of the conditions of electrical stimulation in living protoplasm show a close parallelism with the conditions of electrolysis at metallic electrodes (polar character, existence of threshold intensities, time-relations in the case of brief currents). It has been shown that many organic membranes in contact with electrolyte solutions exhibit properties closely similar to those of metallic electrodes under similar conditions.[3] From these and other facts (e.g., the characteristics of the bioelectric variations) the inference seems justified that in the determination of the chemical reactions of living protoplasm the surfaces of the interfacial films and membranes play a part analogous to that of the surfaces of electrodes in batteries or similar inorganic systems.

That this is the case is also indicated by one of the most characteristic properties of irritable protoplasmic systems, namely their power of rapidly transmitting chemical or metabolic influence to a distance; this property is seen in the propagation of the effects of local excitation to other regions of the cell or cell-system, e.g., in the nervous system. Evidently the basic protoplasmic structure must be of such a kind that 'chemical reactions occurring at one region are capable of influencing other reactions occurring at distant regions of the living system. It is difficult to explain this influence except on the hypothesis that the continuous protoplasmic surfaces have properties of the same general kind as electrode surfaces. Something corresponding

[3] See R. Beutner, "Die Entstehung elektrischer Ströme in lebenden Geweben," Stuttgart, 1920.

to "chemical distance-action" is indicated. It is well known in electrochemistry that processes at one electrode influence those at another when the two electrodes are in metallic connection and immersed in a continuous electrolyte solution. In this case the transmission of chemical influence depends on the flow of current through the circuit, with simultaneous electrolysis at the two electrode surfaces. In living protoplasm the spread of chemical influence is too rapid to be accounted for on the basis of direct transport of material; changes of temperature and pressure are also excluded as transmissive factors. Nothing but the electrical factor seems adequate to produce the observed effect. In mammalian nerve the speed of transmission is 100 meters per second or more, and it is significant that all such rapidly transmitting protoplasmic systems exhibit extreme sensitivity to the electric current, as well as extreme quickness of response.* Such facts suggest that the transmission of chemical effects in protoplasm, as in the inorganic systems just cited, is dependent on the presence of surfaces with electrode-like properties which form circuits with the electrolyte solutions present. These surfaces can only be the surfaces of the protoplasmic structures.

In fact, various inorganic systems are known which exhibit phenomena of chemical transmission strikingly similar to those observed in protoplasm. The transmission of activation in passive metals and similar systems (e.g., mercury in H_2O_2) is an instance; this transmission occurs under conditions resembling closely in their general features those associated with the transmission of excitatory influence in nerve and other irritable protoplasmic systems. What is common in the structure of both systems is the presence of two electrically conducting phases separated by a thin film of chemically alterable material. In the passive iron wire in nitric acid the metal and the acid are the two phases; between the two is a thin (probably monomolecular) film of oxide. Similarly in a protoplasmic structure such as a nerve fiber, the internal protoplasm and the surrounding medium (e.g., lymph) are the two phases; these are separated by the thin surface-film of modified protoplasm or plasma membrane. In both systems the electromotor properties of the surface (interfacial P.D.) are determined by the character of the film.

The susceptibility of both systems to activation by local mechanical, chemical, or electrical influence is directly dependent on the presence and special properties of these interfacial films. Local alterations in a film, e.g., interruptions in its continuity, produce corresponding alterations in the electromotor conditions at the film-covered surface; and the local circuits thus arising have chemical effects which change secondarily the state of other regions of the film at a distance from the altered region. It is well known that a passive iron wire is readily activated by cathodic reduction, and that the transmission of a locally induced activation along the whole length of the wire is a secondary result of this condition. Any area which is rendered active (e.g., by scraping) becomes anodic relatively to the passive area adjoining; hence the latter is immediately activated, cathodically, by the local current flowing between the two areas. By the automatic repetition of this effect the activation spreads over the whole surface. Similarly in a nerve fiber or other irritable protoplasmic system a local alteration of the surface-film induces a local circuit, and apparently the adjacent resting regions are stimulated or activated elec-

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* The electromotor variation in a stimulated mammalian nerve rises to its maximum in 0.0005 second or less.

trically by the current of this circuit. Stimulation involves alteration of the film; hence the effect automatically spreads, just as in the inorganic system. Further comparison between the two systems is not possible in the present brief article; but the parallels are sufficiently detailed to make it almost certain that the same type of process is concerned in both cases.[4]

According to the present view, therefore, chemical reactions occurring at the film-covered structural surfaces of the protoplasmic system, under the polarizing influence of electric currents traversing these surfaces, form the basis of its characteristic irritability. Since this irritability manifests itself in the modification of growth under stimulating conditions (including electrical), as well as of other activities, we may conclude that the synthetic metabolic reactions, besides the other chemical reactions characteristic of living matter, have their chief seat at the protoplasmic interfaces. Further evidence that this is the case is seen in the susceptibility of growth processes, as well as of protoplasmic activity and irritability in general, to reversible suppression by surface-active substances (narcosis by organic compounds), since there is definite evidence that the narcotic action of such substances is based on their effect in displacing the reactive compounds from the structural surfaces.[5]

The resemblance of protoplasmic structure to emulsion structure consists essentially in its being composed largely of finely sub-divided phases or structural elements which are isolated or bounded by thin surface-films. The stability of protoplasmic structure, as of emulsion structure, depends on the properties of these films. A special peculiarity of protoplasm, however, is that typically the phases on either side of a film are *both* aqueous in composition; further, that the films resist the diffusion of water-soluble compounds, i.e., are semi-permeable. This condition is illustrated by any suspension of living cells, e.g., blood; the internal protoplasm and the external medium (serum) are both complex aqueous solutions; between the two is the thin semi-permeable surface-film or plasma membrane. A similar condition is also characteristic of the majority of film-bounded regions in the internal protoplasm of many cells, e.g., vacuoles, cell-inclusions, fibrils and other structures.

It is probable that this condition is mainly responsible for what is most characteristic in the chemical behavior of protoplasm, in particular, for its electrical sensitivity and its power of transmitting chemical influence to a distance. A necessary condition of the latter property (assuming that the foregoing comparison with the passive iron system is a valid one) is that *both* of the adjoining phases should be good conductors of electricity. It is **further** to be noted that the interfacial films besides being difficultly permeable to ions and hence highly polarizable, consist largely (like all protoplasmic structures) of chemically reactive (e.g., oxidizable) material. Hence they are subject to changes of chemical composition, with associated changes in their physical properties, e.g., permeability and electrical polarizability. As already pointed out, this general resemblance to the conditions in electrically sensitive and transmissive inorganic systems, such as the passive iron system, appears to explain why the chemical effects of local alteration are transmitted to a distance.

It has long been known that many kinds of protoplasmic structure are highly unstable; and the recent work on microdissection has shown especially the readiness with which surface-films are built up and broken down in many

[4] See R. S. Lillie, *J. Gen. Physiol.*, **3**, 107, 129 (1920); *Science*, **48**, 57 (1918).
[5] See Warburg, *loc. cit.*

cells.[6] Apparently this instability of the protoplasmic film-structure, combined with its susceptibility to chemical change under electrical influence, is the essential feature determining irritability. The film-bounded structural surfaces of protoplasm have the adsorptive and catalytic properties of electrically polarized surfaces in general (such as the surfaces in charcoal or platinum suspensions); but superposed on these properties are those special features of activity and physical behavior which depend on the variability of the films. This variability carries with it variability of interfacial potentials; such variations readily give rise to local circuits; and these apparently form the basis of many special chemical effects, including the transmissive effects so characteristic of protoplasm. Other general features of protoplasmic action, such as the changes of surface-tension exhibited in contractile processes, are in all probability similarly based on changes in film-structure. Further study of the relation of film structure to the chemical reactivity of the living system may afford insight into other features of its chemical behavior as yet unexplained, in particular the predominance of specific syntheses and the dependent power of maintenance and growth.

[6] For a summary of this work see my book cited above, Chapter 14.

The Nature of the Living Cell as Revealed by Micromanipulation *

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The science of cytology owes its birth to investigators who were primarily experimentalists. When the names of Dutrochet, Dujardin, Brown, Schleiden, von Mohl, Berthold and Pfeffer are called to mind we immediately visualize men who concerned themselves not so much with observations as with methods of experimentation.

The investigations on the cell were seriously hampered, however, by its minuteness and by the apparent homogeneity of its protoplasm. When, therefore, artificial methods were discovered which brought into evidence, in the killed cell, structures of ever increasing complexity, the subject was more and more taken over by the morphologist. Even today the term "cytology" expresses little more than a structural study of killed and stained cells. The pendulum is now swinging back to the experimental method and there is accumulating a wealth of literature which deals with experimental cellular biology and cellular physiology.

Extended studies have been made on the effect of subjecting living cells to different environmental conditions, and our knowledge of the permeability of protoplasm and the action of electrolytes, especially those found in physiological fluids, has been considerably advanced. Most of the results obtained in these studies are based on the indirect method of dealing with masses of cells. The more direct method is to deal with the individual cell.

Owing to the peculiar nature of protoplasm it can be studied only within microscopic dimensions and the most direct method of doing this is to use contrivances which permit manipulation within such confines.

Attempts have long been made to operate on the cell by mechanical means. However, it was not until comparatively recently, between 1904 and 1910, that McClendon, in New York, Barber in Kansas, and Schouten, in Holland, independently of one another, elaborated a technic which enabled one to manipulate with exceedingly fine glass needles and pipettes in the field of the higher magnifications of the compound microscope.

Barber and Schouten used their technic almost exclusively for isolating microörganisms. This was done with micropipettes which were made by carefully breaking off the tips of hollow glass needles. In 1912 Kite used needles with unbroken tips and was the first to apply the technic to a study of the physical properties of protoplasm. Since then the method has been further developed with improvements in the construction of the needles and pipettes and of the instruments for manipulating them.

There are now three forms of micromanipulators which are in most common use,[1,2,3] one of which is shown in Figure 1.

They are all instruments to be used with a compound microscope and are furnished with screws by means of which the movements of microneedles and micropipettes can be accurately controlled in the field of the highest magnifications of the microscope. The needles or pipettes are mounted in special carriers of the instrument and project into a moist chamber on the stage of the microscope. The microtips of the needles and pipettes are made by drawing the end of a glass capillary in a micro flame. The tips of the needles taper to a point of excessive fineness far below one micron (0.001 mm.) in size and the micropipettes for injecting purposes may possess an aperture as small as half a micron in diameter.

The cells or tissues to be dissected or microinjected are placed in a hanging drop of a suitable medium suspended from a thin glass coverslip which roofs the moist chamber.

Recently developed accessories of great importance are microelectrodes [4,5,6] and mechanical devices for microinjection. With such instruments at hand there seems to be no end to the opportunities for experimenting with protoplasm by the direct manipulative method. It lends itself not only to investigations in cellular physiology but to problems in pure physical chemistry, e.g., the physical state and viscosity of emulsions [7] and colloids.[8,9] The method is also being used to advantage in the study of the physiology of urinary secretion [10,11] and of the function of blood capillaries.[12,13]

The results from the micromanipulative technique * on more purely cellular phenomena may be listed under the following headings.

1. EXPERIMENTAL EMBRYOLOGY

Kite,[14] the pioneer in cellular microdissection, did a great amount of work during the few years before his death in 1918. Most of the results he obtained were never published. In a lecture delivered at Woods Hole in 1914 he described graphically an extraordinary operation which he performed on the sperm and egg nuclei of a tropical sea urchin egg whose diameter is less than one-tenth of a millimeter. These results I have since verified on the

[1] Chambers, R., "New apparatus and methods for the dissection and injection of living cells," Anat. Record, 24, 1 (1922). (Reprinted in J. Roy. Micr. Soc., Dec. 1922).
[2] Péterfi, T., "Mikrurgische Methodik," Handb. biol. Arbeitsmeth. (Abderhalden), 5, 479 (1923).
[3] Taylor, V. C., "Improved micromanipulation apparatus," Univ. of Cal. Publ. Zool., 26, 443 (1925).
[4] Ettisch, G. and Péterfi, T., "Zur Methodik der Elektrometrie der Zelle," Arch. ges. Physiol., 208, 454 (1925).
[5] Taylor, C. V., "Microelectrodes and micromagnets," Proc. Soc. Exp. Biol. Med., 23, 47 (1925-26).
[6] Taylor, C. V. and Whitaker, D. M., "Potentiometric determinations of the protoplasm and cellsap of Nitella," Protoplasma, 1927. In press.
[7] Freundlich, H. and Seifriz, W., "Über die Elastizität von Solen and Gelen," Z. physik. Chem., 104, 233 (1923).
[8] Freundlich, H. and Hauser, E. A., "Zur Kolloidchemie der Kautschukmilchsafte," Kolloid Z., 36 (Zsigmondy Festschrift), 15 (1925).
[9] Hauser, E. A., "Über die Anwendung des Mikrurgischen Verfahrens in der Kolloidchemie," Kolloid Z., 38, 76 (1926).
[10] Wearn, J. T. and Richards, A. N., "Observations on the composition of glomerular urine, with particular reference to the problem of reabsorption in the renal tubules," Am. J. Physiol., 71, 209 (1924-25).
[11] White, H. L. and Schmitt, F. O., "The site of reabsorption in the kidney-tubule of Necturus," Am. J. Physiol., 76, 483 (1926).
[12] Florey, H., "Observations on the resolution of stasis in the finer blood vessels," Proc. Roy. Soc., B, 100, 269 (1926).
[13] Landis, E. M., "The capillary pressure in frog mesentery as determined by micro-injection methods," Am. J. Physiol., 75, 548 (1925-26).
* A detailed description of the micromanipulative technique is given in "A Handbook of Microscopical Technique," edited by C. E. McClung and published by Paul B. Hoeber, New York, 1928.
[14] Kite, G. L., "Studies on the physical properties of protoplasm," Am. J. Physiol., 32, 146 (1913).
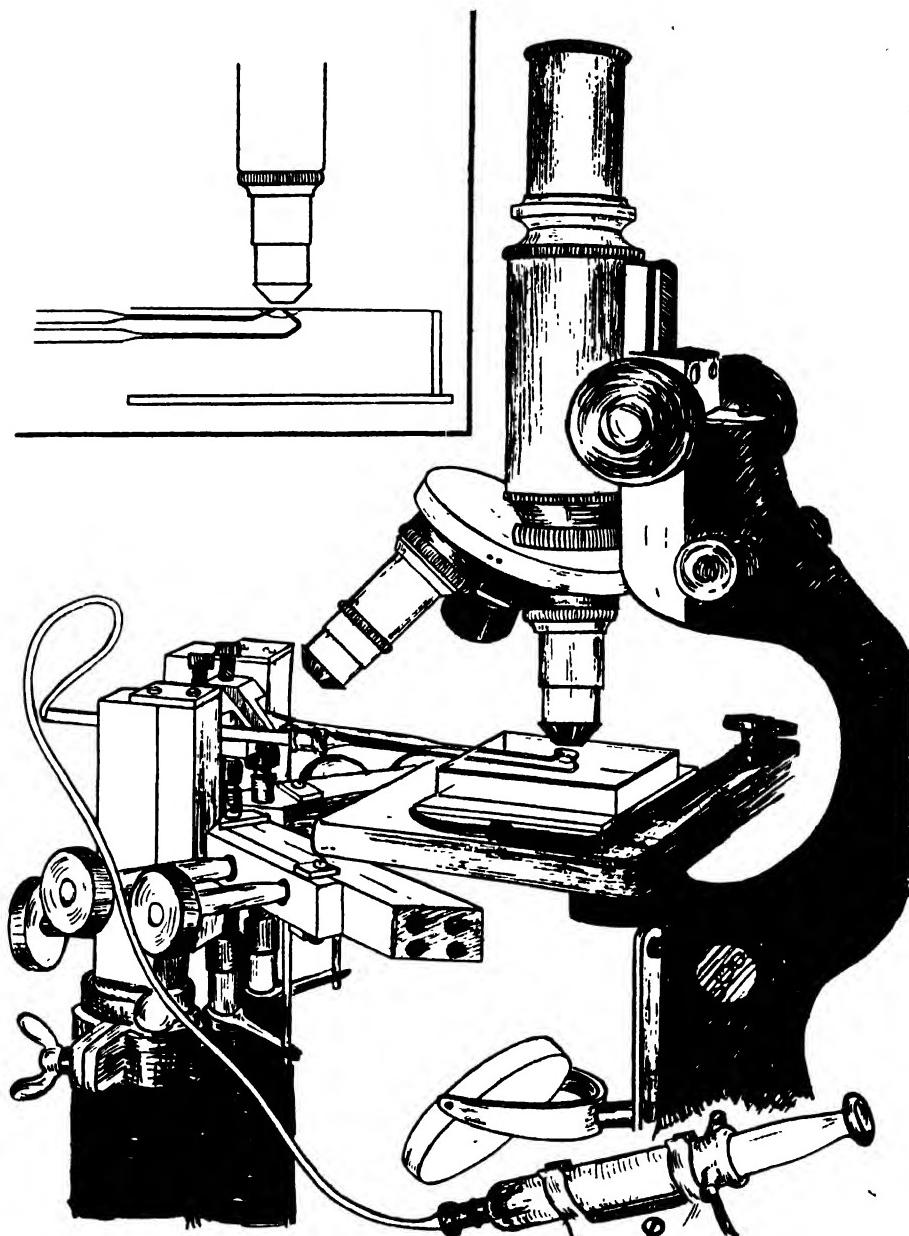
Fig. 1.—Sketch of double micromanipulator placed in front of microscope. One micro-needle and one micropipette are mounted in carriers so that they project into the moist chamber with their upturned tips immersed in a hanging drop containing cells to be dissected and injected. In the foreground is the syringe by means of which the injection is performed. The inset is a diagram giving a sectional side view of the moist chamber with the micro-tips immersed in the hanging drop.

almost equally translucent sanddollar egg. The sperm and egg nuclei are minute spherical bodies considerably less than one-two-hundredth of a millimeter in diameter. Upon insemination of the egg there occurs a march of events not the least remarkable of which is the migration of these nuclei toward each other and their ultimate fusion to form the nucleus of the segmenting egg. Kite attempted to prevent this fusion both by interposing the tip of a microneedle between them and by seizing one nucleus and dragging it away. In each case the nucleus, when released, resumed its normal course. This experiment of Kite's is an illustration of the minuteness of the operations which are made possible by this technic.*

One of the most puzzling phenomena in the segmentation of the egg has been the formation of astral configurations which have been likened to mag-

a   b   c
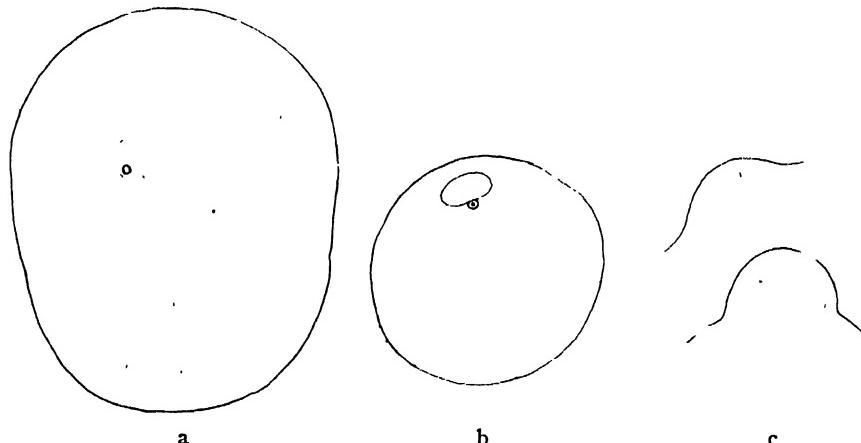
FIG. 2.—a. Amphiaster in an Echinoderm egg being distorted by a microneedle. b. Nucleus pushed from the center to the periphery of an egg. c. Formation of an aster about the dislocated nucleus. (Bibliography 37.)

netic fields of force controlling cell division. By means of microdissection it has been demonstrated that these asters are gelated regions of the protoplasm.[15] It has been possible to move them about within the cytoplasm of the living egg, and, by shifting them to new positions, to change the original plane of cleavage, Figure 2. Drastic mechanical agitation causes them to revert to a fluid state and to disappear, whereupon cell division is prevented.

Among other studies may also be cited the results of removing the nucleus from the egg [16, 17] and the effects of such removal on hybridization.[18]

2. EXPERIMENTAL PROTOZOOLOGY

Experiments on ciliates have demonstrated that the micronucleus is essential for life, and have given proof for the neuromotor function of differentiated

* See also paper by E. E. Just in this volume. *J. A.*
[15] Chambers, R., "Microdissection studies. II. The cell aster; a reversible gelation phenomenon," *J. Exper. Zool.*, 23, 483 (1917).
[16] McClendon, J. F., "Experiments on the eggs of Chaetopterus and Asterias in which the chromatin was removed," *Biol. Bull.*, 12, 141 (1906-07).
[17] Chambers, R. and Ohshima, H., "Merogony experiments on sea-urchin eggs," *Proc. Exp. Biol. Med.*, 19, 320 (1921-22).
[18] Taylor, C. V. and Tennent, D. H., "Preliminary Report on the development of egg fragments," Carnegie Instit. Year Book, No. 23, p. 201, 1924.

fibrils in Euplotes.[19, 20] Evidence is given on the long disputed question of the presence or absence of a true membrane limiting the contractile vacuole of certain protozoa.[21, 22] A microneedle pressed against a contractile vacuole indents its surface, Figure 3a. Vacuoles liberated into the surrounding water by tearing the plasmalemma of the cell with microneedles, Figure 3b, maintain their identity and may be manipulated outside of the cell.

A direct correlation between the amount of water in the endoplasm of an ameba and the volume of water expelled by the contractile vacuole has been demonstrated by microinjection of controlled amounts of water into the animal.[23] The assumption that the vacuole may take up and expel substances in
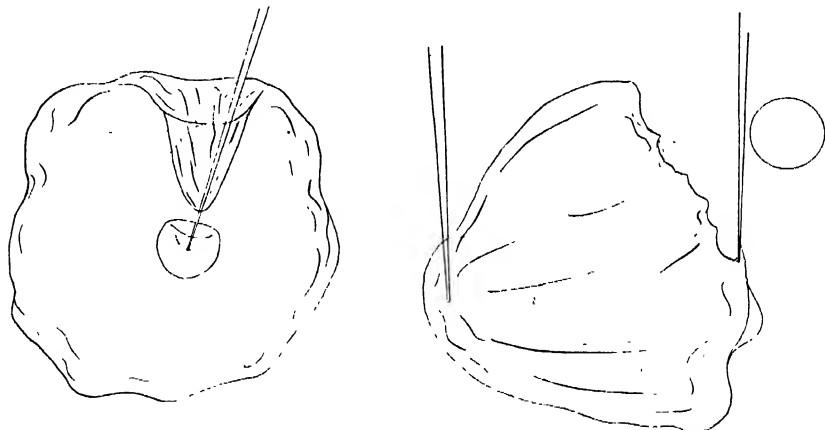
FIG. 3.—a. Contractile vacuole of Amoeba verrucosa being indented by the tip of a microneedle. b. Tough-membraned Amoeba verrucosa held by one needle and torn with the other so that the contractile vacuole was set free. (Bibliography 23.)

solution in the endoplasm is confirmed by injecting a colored solute (picric acid) and following its course.[24] Injection of hydrogen ion indicators into the contractile vacuoles of a large protozoon, Actinosphaerium, show that the vacuolar fluid in this form [25] has a pH value of 7.4 ± 0.1.

Microinjection of a minute quantity of a substance (urea) which produces a pronounced fluidity of the endoplasm in the ameba, has been shown to prolong the diastolic interval in vacuolar contraction.[26] Since a normally operating vacuole is known to lie in a gelated region, the extreme dilatation of the vacuole

[19] Taylor, C. V., "Fatal effects of the removal of the micro-nucleus in Euplotes," Univ. Cal. Publ. Zool., 26, 131 (1924).
[20] Taylor, C. V., "Demonstration of the function of the neuromotor apparatus in Euplotes by the method of microdissection," Univ. Cal. Publ. Zool., 19, 404 (1920).
[21] Taylor, C. V., "The contractile vacuole in Euplotes: an example of the sol-gel reversibility of cytoplasm," J. Exper. Zool., 37, 259 (1923).
[22] Howland, R. D. "Experiments on the contractile vacuole of Amoeba verrucosa and Paramoecium caudatum," J. Exp. Zool., 40, 231 (1924).
[23] Howland, R. B. and Pollack, H., "Micrurgical studies on the contractile vacuole," I and II, J. Exp. Zool., 48, 441 (1927).
[24] Howland, R. B. and Pollack, H., "Expulsion of an injected solute by the contractile vacuole," Proc. Soc. Exp. Biol. Med. (1927). In press.
[25] Howland, R. B. and Pollack, H., "The pH value of the contents of the contractile vacuole," J. Exp. Zool. (1928). In press.
[26] Howland, R. B. and Pollack. H., "Microinjection of urea into the protoplasm of Amoeba dubia," Proc. Soc. Exp. Biol. Med., 24, 378 (1927).

subsequent to this experimentally produced fluidity indicates the importance of gelation in normal contraction phenomena.

Our knowledge of the physiology of protein digestion in the protozoa has been extended by the use of the micrurgical method. Microinjection of indicator dyes into gastric vacuoles during the various phases of digestion, show that an acid is secreted into them by the surrounding cytoplasm.[27] The pH value of the vacuolar contents during the period of most pronounced activity is 4.3 ± 0.1.

3. EXPERIMENTAL HISTOLOGY OF SURVIVING TISSUES

The physical state and relationships of various metazoan cells have been investigated and the existence of definite intercellular bridges between the cells of the human epidermis has been definitely established.[28] Cutting and tearing experiments also have been made on cells in tissue culture.[29 to 82]
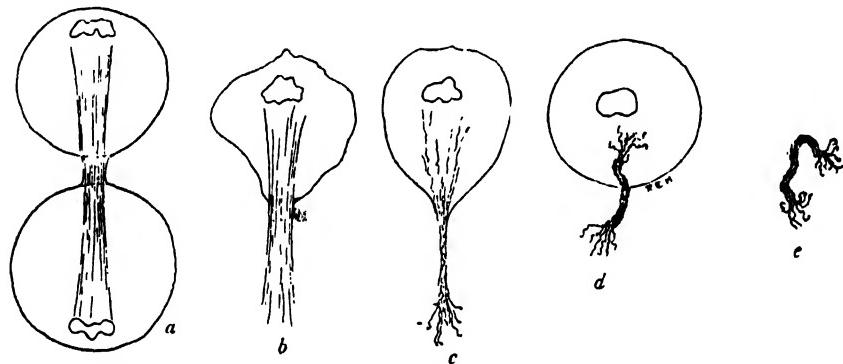
FIG. 4.—a. Dividing germ cell of grasshopper with mitochondrial strands extending between the two daughter nuclei. b. One of the daughter cells has been destroyed by puncturing it with a microneedle. The mitochondrial strands persist. c and d. The strands soon wrinkle and shorten d. The persisting wrinkled mass of mitochondrial strands persisting after the remaining cell has been destroyed. (Bibliography 37.)

The structure and mode of action of cilia and cirri[33, 34] of ciliated epithelium have also been investigated. Carter[33] has shown that at least the larger cilia, such as the latero-frontal cilia of the gills of Mytilus, are complex, being composed of a number of simpler elements, slender triangular plates, each one of which beats independently when isolated. A peculiar structural feature of the cilium is brought out by the fact that when it is stopped with a microneedle during the effective beat, it is clearly quite stiff. During the recovery beat, however, it is so limp that it frequently slips below the needle.

[27] Howland, R. B., "The pH of gastric vacuoles," *Protoplasma* (1928). In press.
[28] Chambers, R. and Rényi, G. S., "The structure of the cells in tissue as revealed by microdissection and the physical relationship of the cells in epithelia," *Am. J. Anat.*, **35**, 385 (1925).
[29] Péterfi, T. and Olivo, O., "Ricerche di microdissezione su cellule somatiche coltivate in vitro," *Compt. rend. Assoc. Anat.*, Reunion, 20 (1925).
[30] G. Levi, "Ricerche sperimentali sovra elementi nervosi, coltivati in vitro," *Arch. exper. Zellforsch.*, **2**, 244 (1926).
[31] Péterfi, T. and Olivo, O., "Die Wirkung des Anstechens auf das Protoplasma lebender Zellen. I and II," *Arch. exper. Zellforsch.*, **4**, 149 and 155 (1927).
[32] Péterfi, T., "Die Mikrurgie der Gewebekulturen," *Arch. exper. Zellforsch.*, **4**, 165 (1927).
[33] Carter, G. S., "On the structure and movements of the laterofrontal cilia of the gills of Mytilus," *Proc. Roy. Soc.*, B, **96**, 115 (1924).
[34] Chambers, R. and Dawson, J. A., "The structure of the undulating membrane in the ciliate Blepharisma," *Biol. Bull.*, **48**, 240 (1925).
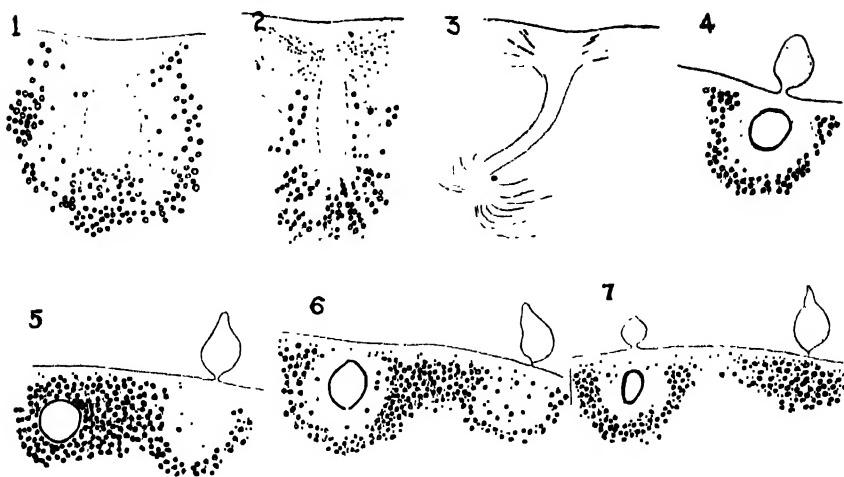
Fig. 5.—1. Periphery of Cerebratulus egg showing early nuclear spindle preparatory to formation of 1st polar body. 2. Nuclear spindle with astral radiations at its two poles. 3. Spindle at stage of '2' stretched by needle in attempting to pull it away from the egg periphery. The peripheral aster is anchored to the surface of the egg. (Bibliography 15.)

Intracellular structures, viz., the nuclear spindle, the mitochondria and chromosomes have been microdissected.[35, 36, 37] The mitochondria at certain

Fig. 6.—Metaphase chromosomes from a pollen mother cell of the Tradescantia, isolated and dissected with microneedles. a. Two isolated tetrad-like chromosomes. b. Half of a chromosome secured by tearing in two the quadruple form shown in upper part of figure in a. c. The half chromosome of the previous figure caught at its two ends by microneedles and stretched. (Bibliography 35.)

stages are exceptionally resistant fibrils which can be set free by destroying the cells in which they are found, Figure 4. The spindle is a formed body

[35] Chambers, R. and Sands, H. C., "A dissection of the chromosomes in the pollen mother cells of Tradescantia virginica," *J. Gen. Physiol.*, 5, 815 (1923).
[36] Chambers, R., "Etudes de microdissection. IV Des structures mitochondriales et nucleaires dans les cellules germinales mâles chez la Sauterelle," *Le Cellule*, 35, 107 (1925).
[37] Chambers, R., "The physical structure of protoplasm as determined by microdissection and injection." Section V, General Cytology, University of Chicago Press, 1924.
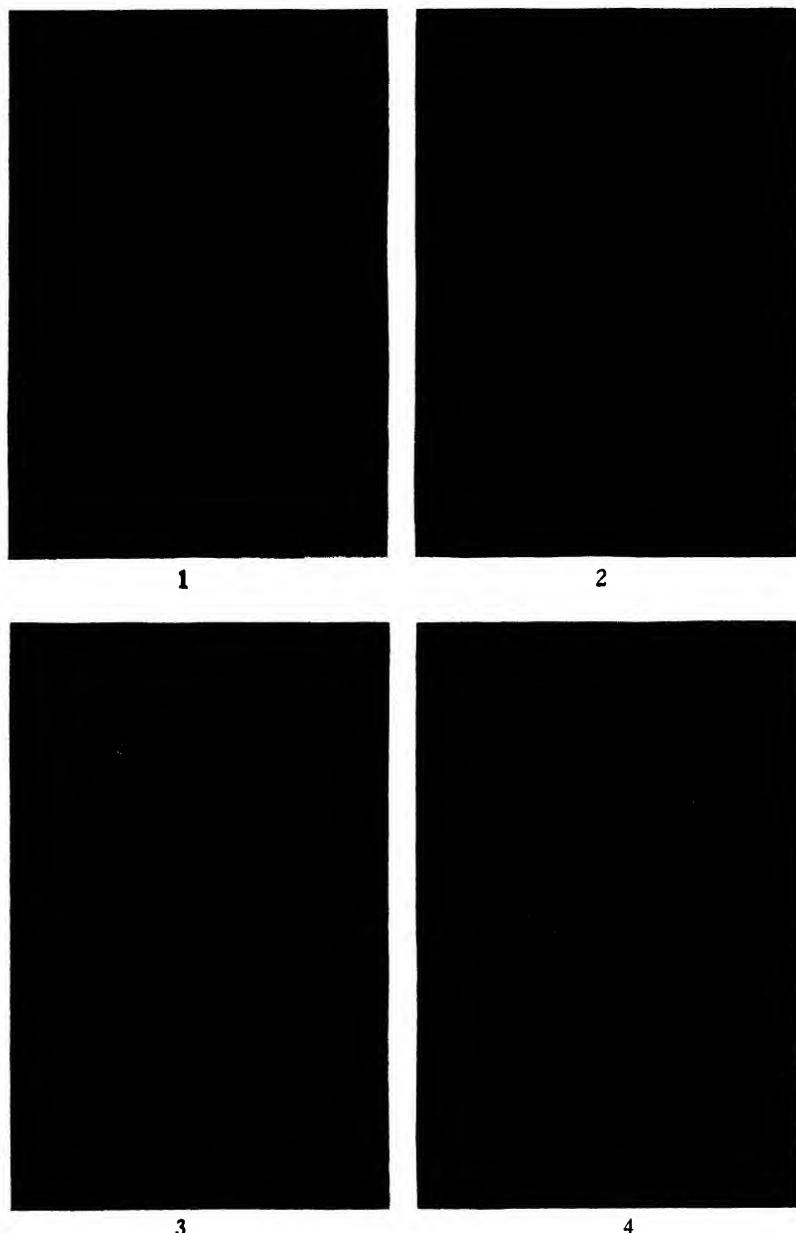
FIG. 7.—1. A strand pulled out from a naked protoplasmic surface of a starfish egg. 2. On being released from the needle, the strand flows back into the protoplasmic mass with no sign of its having been formed. 3. A stretched strand after being held for some time breaks into several coherent droplets. 4. Major part of the strand has broken away and has flowed back into the protoplasmic mass, leaving two droplets on the tip of the microneedle.

suspended in an optically structureless matrix. When the vacuoles are numerous and crowded together the protoplasm is frothy and tends to be firm in consistency. The consistency of protoplasm may also vary through changes in the hyaline matrix which are analogous to those occurring in the formation of sols and gels in colloids. The ultramicroscopic appearance of this matrix and the reversibility of its physical state are some of the properties by virtue of which it is classified as a colloid.

Evidence from experiments on the injection of aqueous and non-aqueous solutions into such diversified cells as the ameba, the pancreas cell of the frog, and marine ova, clearly indicates that their internal protoplasm is freely miscible with aqueous solutions. In small quantities water diffuses readily through the protoplasm without affecting its viable state. In larger quantities it induces destructive changes.[39]

The injection of solutions of the chlorides of sodium, potassium, calcium and magnesium has thrown considerable light on the importance of electrolytes in protoplasmic functions. The salts of the monovalent electrolytes, sodium and potassium increase the dispersion of the internal protoplasm. This is similar to their effect on the plasma membrane. On the other hand, the injection of solutions of $CaCl_2$ or of $MgCl_2$ produces a coagulation. This is in striking contrast to their effect on the plasma membrane upon which they have no coagulating action.[39]

These phenomena emphasize two facts of great significance concerning the action of electrolytes on protoplasm. One fact is the profound difference between the plasma membrane and the internal protoplasm (Fig. 8). The other fact offers an explanation for the mutual antagonism of mono and bivalent cations both on the internal protoplasm and on the plasma membrane, viz., the normal physical state of the internal protoplasm can be maintained when the bivalent and monovalent cations are present in certain proportions because the solidifying action of the one will balance the dispersing action of the other. Similarly the antagonistic action of the electrolytes on the plasma membrane is due to the balance between the stabilizing action of the bivalent and the dispersing action of the monovalent cations.

This fact is in harmony with the interpretation of protoplasmic systems given by Clowes in his experiments on the action of salts on water and oil emulsions.[40] The calcium salts favor the formation of an emulsion of water in oil and would therefore enhance the maintenance of a lipoid-like film on the surface of protoplasm. The toxic action of the sodium salts on such a film is due to their dispersive action on the lipoid by breaking it up into an emulsion of oil in water.

Upon the intact condition of the plasma membrane depends the integrity of the interior of protoplasm and the very salts which are least injurious to the surface and upon which the surface most depends for its maintenance are the salts which are most injurious to the interior.

In the absence of salts the protoplasm of Echinoderm eggs coagulates. This can be shown by plunging the eggs with a, minimum of sea water into a large quantity of distilled water. The eggs begin to swell by osmosis but before the surface breaks the entire mass of the egg sets into an irreversible coagulum.

We may state, therefore, that the salts maintain the peculiar colloidal state characteristic of living protoplasm, and the relative amounts of the two types

[40] Clowes, G. H. A., "Protoplasmic equilibrium," *J. Phys. Chem.*, **20**, 407 (1916).

a   RECOVERY OF AMOEBAE FROM INJECTION OF DECREASING CONCENTRATIONS
OF SALT SOLUTIONS (NACL, KCL, CACL₂ & MGCL₂)

b   VIABILITY OF AMOEBAE IN DECREASING CONCENTRATIONS OF
SALT SOLUTIONS (NACL, KCL, CACL₂ & MGCL₂)
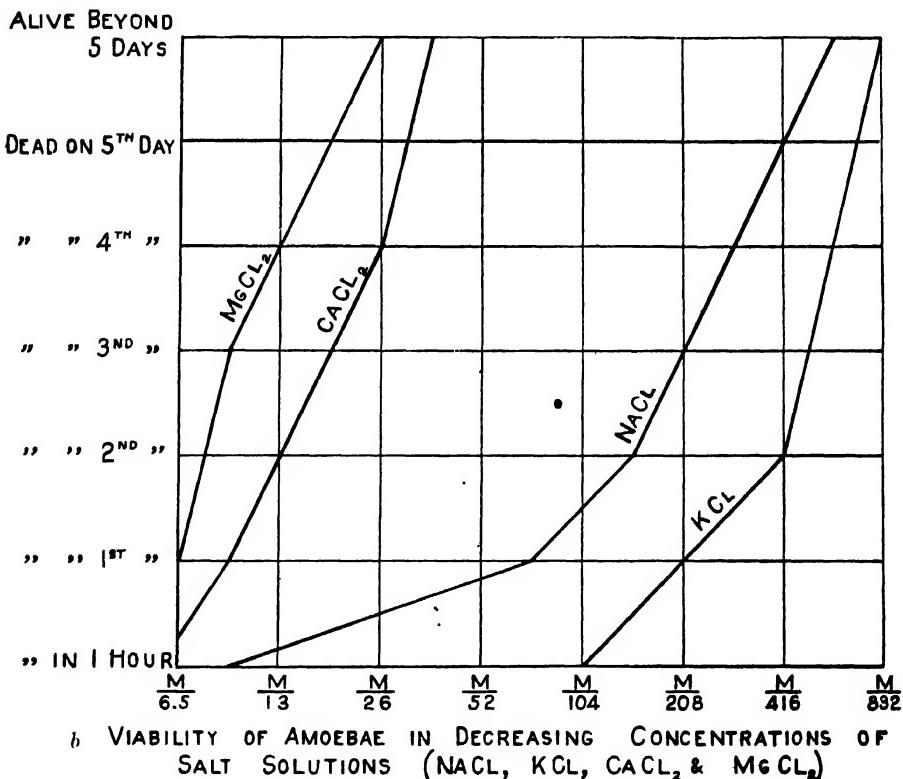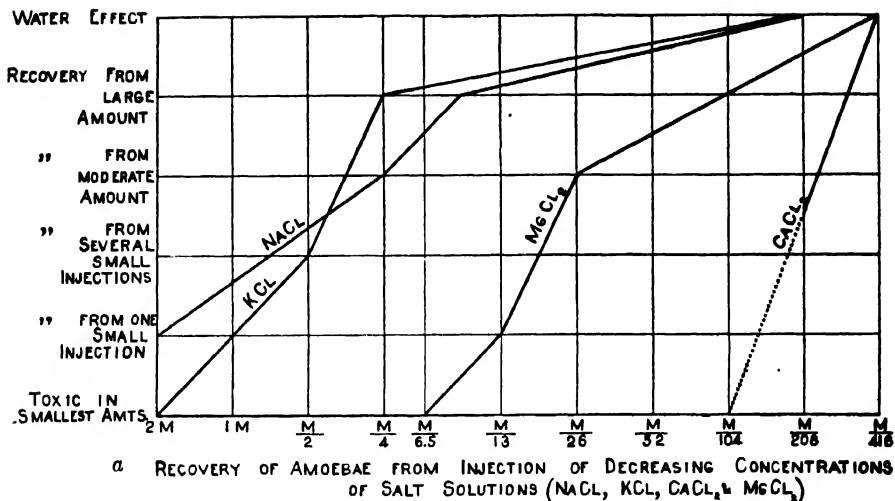
FIG. 8.—Two charts to show the reversed order of toxicity of the chlorides of the
monovalent and of the divalent cations when applied to the interior (chart a) and
to the surface (chart b). (Bibliography 39.)

of salts present are so proportioned that the associative or solidifying action of one is offset by the dispersive action of the other.

The pH of Protoplasm

The importance of acid-base conditions on the action of electrolytes and on protoplasmic activities in general led to a study of the hydrogen ion concentration of the cell interior. A considerable amount of work has been done on this problem without the aid of the micromanipulation technique. Of the published data, however, comparatively few can be considered to deal strictly with the intraprotoplasmic pH. The results of even these few are open to criticism because of the possibilities of error from injury to the tissues in the procedures employed.

A case in point is the use of fluid extracts from crushed tissues. Not only is there a possibility of error from crushing cellular tissues but also from mixing of intracellular extracts with the highly buffered extracellular fluids which can never be completely eliminated from a mass of tissue. There are also serious objections in attempts to determine pH by exposing cells to solutions of indicator dyes either by immersion or by injecting the dyes into the circulation. One of the objections is the impermeability of normal living cells to the vast majority of useful indicator dyes. The other is the fact that those dyes which do penetrate, e.g., neutral red, almost always become adsorbed on or accumulate within intracellular inclusions, such as granules and vacuoles, and give variable indications irrespective of the true pH of the protoplasmic substratum.

By means of the micromanipulative technique both electrometric and colorimetric methods are conceivably possible for determining the pH of protoplasm. The electrometric method is still in the experimental stage.

Definite results have been obtained with the colorimetric method by injecting solutions of indicator dyes into the protoplasm. The new Clark and Lubs series of indicators and the basic dyes, neutral red and methyl red, have proved to be most serviceable for this purpose. They are prepared in aqueous solutions of sodium salts, and, when injected into the protoplasm, readily permeate it. This is in accordance with results already obtained with other sodium salts [39] which maintain the fluid state of the protoplasm and allow the ready diffusion of the injected solution throughout the cytoplasm. Fortunately the dyes are relatively non-toxic. This is specially true for phenol red, the useful pH range of which happens to include the pH value found for protoplasm. Amebae, for example, after the injection, maintain the color of the dye and live in a normal condition for days. An important consideration regarding the validity of the colorimetric method for determining the intraprotoplasmic pH is the fact that all the solutions of dyes, both acid and basic, have been found to give consistent indications, when injected, irrespective of whether the dissociation of the dyes is at its lowest in their acid or alkaline ranges.[41]

Colorimetric determinations by means of microinjection have already been reported by several investigators. Schmidtmann [42] introduced solid granules of dyes into mammalian cells where the granules went into solution and gave characteristic color reactions. Her findings of the pH in the cells of different

[41] Chambers, R. and Pollack, H., "Micrurgical studies in cell physiology. IV. Colorimetric determination of the nuclear and cytoplasmic pH in the starfish egg," *J. Gen. Physiol.*, **10**, 739 (1927).

[42] Schmidtmann, M., "Über die intracellulare Wasserstoffionenkonzentration unter physiologischen und einigen pathologischen Bedingungen," *Z. ges. exp. Med.*, **45**, 714 (1925).

tissues give values varying from 6.3 to 7.5. It is too early to decide to what extent these variations are due to the presence of secretory products in the cells rather than to inherent differences in the true protoplasmic pH.

The Needhams [43] injected solutions of dyes of the Clark and Lub's series into Amoeba proteus and recorded its pH to be between 7.4 and 7.6. From experiments conducted in this laboratory we place the pH of the ameba at 6.9 ± 0.1. In our experiments the injected amebae survived the injection and maintained the characteristic color for days. The fact, therefore, that the amebae injected by the Needhams did not survive longer than several minutes, suggests that the high pH which they obtained may be due to some toxic effect.
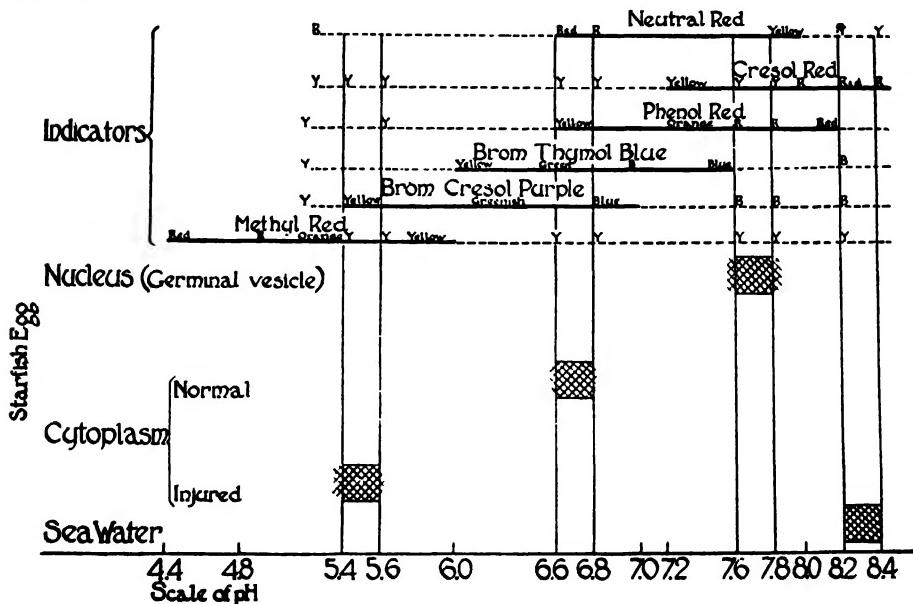
FIG. 9.—Tabular representation of the pH of the healthy and injured cytoplasm and of the nucleus of the immature starfish egg. Aqueous solutions of the indicators were successively injected into different eggs and the assumed colors are indicated by the positions of the vertical columns. (Bibliography 45.)

The most unequivocal results obtained so far are those on the starfish egg in which our findings [41] agree with those of the Needhams [44] in placing the cytoplasmic pH at a figure within one or two decimal points of 6.8, Figure 9.

We have also determined the pH of the living nucleus to lie between 7.6 and 7.8. This determination was made on the germinal vesicle of the living starfish egg, which, after the injection, underwent normal changes preparatory to maturation, Figure 10.

Recently we have used the same method on cells from the body of the frog and Necturus, e.g., active and resting muscle, epithelial and glandular

[43] Needham, J. and D. M., "The hydrogen-ion concentration and the oxidation-reduction potential of the cell-interior: a micro-injection study," *Proc. Roy. Soc.*, B, **98**, 259 (1925).
[44] Needham, J. and D. M., "The hydrogen-ion concentration and oxidation-reduction potential of the cell-interior before and after fertilisation and cleavage: a micro-injection study on marine eggs," *Proc. Roy. Soc.*, B, **99**, 173 (1926).

cells.[45] The injection of these cells indicates a fairly constant pH value of 6.8 to 7.0 for the cytoplasm and 7.6-7.8 for the nucleus.

All these experiments point to the probability that the cytoplasm of living cells in general has a reaction which is constant within very narrow limits. The nucleus of different types of cells also shows a similar uniformity, viz., a constant hydrogen ion concentration of about 7.6 to 7.8. Another fact of interest is that the pH of the protoplasm of cells is distinctly more acid than their surrounding media. For example, the marine ova with an intracyto-

Fig. 10.—Photograph of a starfish egg undergoing maturation after its cytoplasm and nucleus had been injected with the pH indicator, phenol red. 1. Immature egg held by a diagonal needle and with the tip of the vertical micropipette in the nucleus where it is seen as a black spot. The nucleus is red and the cytoplasm yellow. 2. Egg, one hour later, lying in a deeper region of the hanging drop. The nucleus has begun to shrivel and the cytoplasm is taking on an orange tint. 3. Egg, 10 minutes later, with yellow cytoplasm. The diminutive pronucleus prior to polar body formation can be seen near center of egg. (For an interpretation of the color tints of phenol red in terms of pH, see Fig. 9.) (Bibliography 45.)

plasmic pH of about 6.8 live in sea water the pH of which is about 8.4. Metazoan tissue cells are bathed in tissue fluids and blood which have a pH of about 7.5 and fresh water amebae thrive best in water having a slightly alkaline reaction.

THE OXIDATION-REDUCTION POTENTIAL OF PROTOPLASM

The micromanipulative method has also made possible an investigation of another most important phase of cellular activity, viz., the oxidation-reduction potential of protoplasm. A determination of this factor will probably prove to be of great value in a study of the respiration and metabolism of the cell.

Clark, Cohen and coworkers[46] have recently elaborated and standardized a number of indicator dyes which differ in the ease with which they can be oxidized and reduced. These dyes are arranged in a series in the order of their ease of reduction (in terms of the electrode potential of their equilibrium system). The dyes are mainly the indophenols and the indigotines. The indophenols constitute a majority of those dyes which are more easily reducible than methylene blue while the indigotines are reduced with greater difficulty. As a measure of reduction intensity Clark chose the symbol rH

[45] Chambers, R., Pollack, H. and Hiller, S., "The protoplasmic pH of living cells," *Proc. Soc. Exp. Biol. Med.,* 24, 760 (1927).
[46] Clark, Cohen and co-workers, "Studies on oxidation-reduction. I-VIII," *U. S. Public Health Reports,* Nos. 38, 39, 40 (1923-25).

which is the logarithmic value of the hypothetical molecular hydrogen pressure in equilibrium with the oxidation-reduction system in question. Just as pH refers to the intensity of acidity in distinction to the total amount of acid present, so rH refers to the intensity of reduction in distinction to the total amount of reductant or oxidant present. The computed rH value has definite significance only when the pH is constant for any set of comparisons.

Therefore, in order to determine the rH or the oxidation-reduction intensity of a system one must know not only which of the dyes are completely, partially or not at all reduced but also the pH of the system at the time when the determination is being made.

To the Needhams of Cambridge, England,[43, 44, 47] is due the credit of realizing the possibility of determining the intraprotoplasmic rH by means of microinjection. They selected, as material, fresh water amebae and the eggs of starfish and sea-urchins. They determined the rH of ameba to be 17-19 and that of the eggs to be 21-22. Rapkine and Wurmser [48] made similar studies on salivary gland cells of insect larvae and echinoderm eggs whose rH they placed between 19 and 20.4. Thus, these investigators have been able to demonstrate the existence of a definite degree of the intensity factor for oxidation-reduction in protoplasm. In other words the oxidation-reduction activity appears to be poised at a fairly constant level quite analogous to the maintenance of the hydrogen-ion concentration in a well buffered system. Moreover the intensity factor can be expressed in terms of quantitative values. Their results indicate that this intensity factor differs in different cells for it appears to be distinctly greater in the fresh water ameba than in the marine starfish egg. According to the Needhams the oxidation-reduction intensity in anaerobic protozoa is greater than that in aerobic forms, such as the ameba. However, they found that in the latter the intensity is maintained at the same poised level under both aerobic and anaerobic conditions. This is rather extraordinary when considered in the light of the observations of Cannan, Clark and Cohen [48] on cell suspensions in which anaerobic conditions pronouncedly change the rH.

More recently a group of us have made an intensive study of both the pH and the rH of the fresh water ameba and the starfish egg.[49, 50]

It was found that as long as the injected cell remains alive the pH of any solution injected quickly changes to that of the protoplasm of the cell. This important feature made it unnecessary to have the pH of the solutions of the oxidation-reduction dyes accord exactly with that of the cells to be injected. However, because of the fact that the viability of the cells is best maintained when the pH of the injected solution approximates that of the cell the solutions were all made at a pH of 7.0.

The results of our investigations confirm those of the Needhams in establishing a definite level of oxidation-reduction intensity for the living cell in an atmosphere of air. However, our results show that the level is the same for both the ameba and for the starfish egg. If, however, the cells are exposed to purely anaerobic conditions by placing them in an atmosphere of pure

[47] Needham, J. and D. M., "Further micro-injection studies on the oxidation-reduction potential of the cell-interior," *Proc. Roy. Soc.*, B, **99**, 383 (1926).
[48] Cannan, R. K., Clark, W. M. and Cohen, B., "Studies on Oxidation-reduction. X, Reduction potentials in cell suspensions," *U. S. Public Health Reports*, No. 55, p. 1 (1926).
[49] Cohen, B., Chambers, R. and Reznikoff, P., "Intracellular oxidation-reduction studies. I. Reduction potentials of *amœba dubia* by microinjection of indicators." *J. Gen. Physiol.*, in press (1928).
[50] Chambers, R., Pollack, H. and Cohen, B., "Intracellular oxidation-reduction studies. II. The oxidation-reduction potential in Echinoderm eggs (1928)

nitrogen, the level of oxidation-reduction intensity drops to a value closely corresponding to that already found by Cannan, Clark and Cohen [48] in cell suspension under anaerobic conditions. It was also found that an exposure of living cells to an atmosphere of oxygen shifts the level of oxidation-reduction intensity in the opposite direction.

These results, contrary to those of the Needhams, indicate that the oxidation-reduction factor in the living cell is largely a funciton of atmospheric conditions. In other words there seems to be no constancy in the maintenance of a definite oxidation-reduction potential in the living cell other than that determined by the environmental conditions.

CALCIUM IONS IN PROTOPLASM

Pollack has found [51] that the presence of calcium in protoplasm can be demonstrated by the formation of a red insoluble calcium alizarinate when a solution of sodium alizarin sulfonate is injected into the protoplasm of the ameba. The reaction which occurs subsequent to such an injection closely resembles that which occurs when sodium or potassium salts are injected. This leads one to suspect that there is a relation between the results of an increase in monovalent ions and a decrease in calcium ions. Moreover, the observations on the toxic effects of injecting phosphate, carbonate and sulfates [52] have been extended by Pollack to include the tartrate and oxalate. Their toxicities fit in with Pollack's interpretation that the toxic action is due to the removal by these anions of the calcium in the protoplasm. The relative toxicity of these anions is a function of the insolubility of the corresponding calcium salt.

The first effect of injecting calcium precipitants into an ameba is temporary, complete quiescence. Furthermore, there is no return of normal movements until the calcium becomes available to the protoplasm. This is an interesting confirmation of Pantin's experiments [53] in which it was found that marine amebae become motionless if the medium in which they are immersed lacks the calcium ion.

CELL DIVISION

There is still another field of cellular activity which is amenable to investigation by means of the micromanipulative technique. Very little is known concerning the factors which control cell division, an explanation of which should go far toward a solution of some of the most pressing problems in medicine. Before we can hope for much we must build up an adequate conception of the varied physiological processes of the cell, some of which have been reviewed in this article.

The existence of definitely directed currents of flow in the cytoplasm of the segmenting ovum has already been described by several investigators who have suggested that the formation of the cleavage furrow is due to vortical movements in the equatorial region of the egg (cf. 37). These currents are best seen in rapidly dividing cells such as the nematode egg. In the more slowly dividing sea-urchin egg they are barely appreciable but can be strik-

[51] Pollack, H., "Micrurgical studies in cell physiology. VI. Calcium ions in protoplasm," *J. Gen. Physiol.*, in press (1928).
[52] Reznikoff, P., and Chambers, R., "Micrurgical studies in cell physiology. III. The action of CO_2 and some salts of Na, Ca and K on the protoplasm of Amoeba dubia," *J. Gen. Physiol.*, 10, 731 (1927).
[53] Pantin, C. F. A., "On the physiology of amoeboid movement. III. The action of calcium," *Brit. J. Exp. Biol.*, 3, 275 (1926).

ingly demonstrated by taking a time exposure of an egg which has been properly oriented to the lens of a camera, Figure 11. The developed picture exhibits numerous curved streaks caused by the movement of the cytoplasmic granules along the paths of the currents. These currents flow over the surface of the egg from the two opposite poles. At the equator the two opposing currents turn inward producing a vortex which is concomitant with the sinking in of the equatorial furrow. This phenomenon resembles that of an oil

Fig. 11.—Photomicrograph with a time exposure of 4 seconds of a segmenting Echinoderm (Sand-dollar) egg. The egg was held in place with microneedles at its two poles. For description see text.

drop which is made to divide by lowering the surface tension at two opposite poles, and has led some investigators to suggest that surface tension forces play an important rôle in cell division.

The furrow which forms at the equator of the oil drop divides the drop in two only when the two daughter droplets are free to move apart. If, on the other hand, the opposing surfaces of the deepening furrow become contiguous they merge, and the droplet resumes its original spherical shape. Such is not the case in the normally dividing cell, for the furrow frequently advances with its sides in such close contact as to give the appearance of a thin

line. This dissimilarity between the dividing oil drop and the cell has been used as an argument that a comparison between the two is unjustified.

It is of interest to note that the sea-urchin egg can be placed under conditions in which this dissimilarity is largely eliminated. This is brought out in the following experiment in which eggs were allowed to segment in a solution of the salt of a monovalent cation, potassium chloride, isotonic with sea water. As the cleavage furrow deepens, the two daughter cells roll apart, in a manner similar to the behavior of the two halves of a dividing oil drop. If the egg is held with needles so that the daughter halves cannot roll apart, the sides of the furrow between them become contiguous and merge, and the egg remains undivided. The cytoplasmic currents continue unchecked, so that the furrow may repeatedly reappear and disappear.
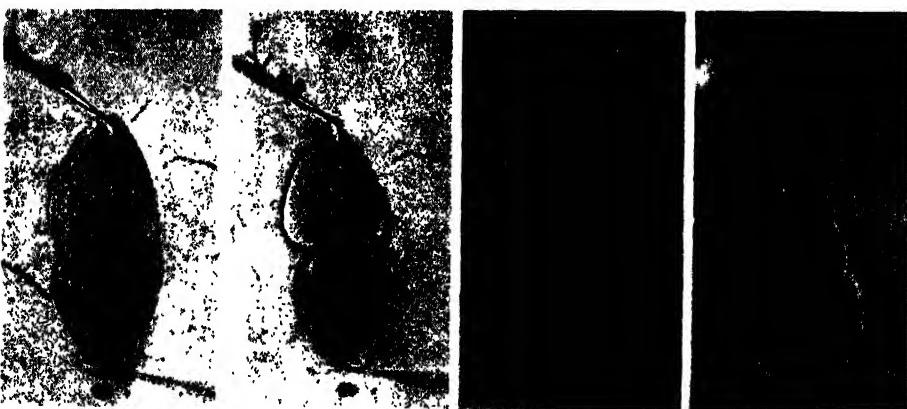
FIG. 12—Four photomicrographs of an Echinoderm egg undergoing segmentation while stretched between two microneedles. For description see text.

The division of the oil drop is initiated by lowering the surface tension at the two poles of the drop with respect to its equator. This is done by the application of sodium carbonate simultaneously at the two poles.[54] In the case of the egg cell normal division is always preceded by definite karyokinetic changes of the nucleus, accompanied by the formation of astral radiations in the cytoplasm. When once these changes have taken place, no mechanical disturbance, short of that which actually destroys the asters, will impede the division of the cell.[37] However, if we exclude the factors which initiate the cytoplasmic currents and take into consideration only the part taken by the currents in the division of the cell, the resemblance between the dividing oil drop and the egg in potassium chloride is strikingly close.

An experiment which strikingly demonstrates the flow of material from the poles to the equator is to divest an ovum of its enveloping fertilization membrane and, when it is just beginning to segment, to seize it with two microneedles at its poles and to stretch it in the direction of its long axis. The needles are then left stationary with the egg stretched between them, Figure 12. In spite of the constrained position the process of division con-

[54] Spek, J., "Oberflächenspannung als eine Ursache der Zellteilung." *Arch. Entwicklm.*, **44**, 5 (1918).

tinues. The egg gradually pulls itself together away from the needles while the segmentation furrow steadily deepens until it has divided the egg into two.

If the two daughter parts of a segmenting egg are held and gradually pulled apart while the furrow deepens, the opposite walls of the furrow will have an appreciable space between them. The superficial currents, curving medianward in the walls of the furrow, can then be more distinctly detected. When the furrow has almost but not quite completed the division, its rate of advance appreciably slackens. Consequently, a narrow cylinder of cytoplasm remains for a time as a stalk between the two blastomeres. This stalk gradually narrows and eventually breaks, which results in a complete separation of the blastomeres.

The variation between the behavior of eggs immersed in potassium chloride and those in their normal environment of sea water containing calcium may be accounted for as follows. The protoplasmic surface of the echinoderm egg is coated with a thin layer of glutinous jelly, secreted by the egg. This is the pellicle which, as mentioned in the earlier part of this lecture, is more or less universally present on living cells. Any newly formed surface, e.g., the sides of the cleavage furrow, exudes a substance which, in the presence of its normal, calcium-containing environment, immediately sets to form a jelly. In the absence of the bivalent cations, calcium and magnesium, the material either dissolves or is softened and washed away. In a solution of potassium chloride, therefore, the egg can divide only when the opposite sides of the furrow do not come into contact. Otherwise, there is nothing to prevent their merging with the consequence that the furrow disappears. In normal sea water, on the other hand, the furrow fills with a jelly as fast as it sinks into the egg. The jelly separates the protoplasmic surfaces bordering the furrow, and prevents their mergence, thus enabling the cell to divide. This phenomenon lines up with our knowledge concerning the need of calcium for the growth of tissues in general. We can now see that in the growth process, one of the requirements which is filled by calcium is to stiffen the intercellular substance* This increases the chances of cell division and also enables the cells to adhere so as to form a coherent tissue.

This article is a statement of the varied phases of cellular activity which are being dealt with by a new method of approach. Any adequate concept of the life processes requires a thorough knowledge of the action of the essential electrolytes not only on protoplasm as a whole, but on its various constituents. In addition to this, and intimately related to it, is the need for a comprehension of the acid-base equilibria and the oxidative processes in the protoplasmic system. All of these contribute to the maintenance of the structural and functional integrity of protoplasm. Through the micrurgical technique we have a direct method for attacking these problems. It offers an opportunity to develop such a conception of the dynamic living cell as has not heretofore been possible.

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* An analogous rôle is played by calcium in soils. A certain amount of calcium humate is necessary to keep soils in good tilth. "Alkali soils" tend to wash away, and are heavy or slimy. *J. A.*

Amoeboid Movement and Agglutination in Amoebocytes of Limulus and the Relation of These Processes to Phagocytosis, Tissue Formation and Thrombosis

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Certain types of unicellular organisms have the ability to send out processes, called pseudopodia; subsequent movements of the remaining parts of the cell in the direction of the pseudopod lead to locomotion of the whole cell. This process depending on changes in form of the cell represents amoeboid movement.

The earlier investigators of amoeboid movement in protozoa noticed a similarity between the sending out of pseudopodia and the movement of drops of fluid under the influence of a localized lowering of surface tension. As a result of a change in surface tension in a drop, the fluid streams forward in the center and flows backward again in the periphery of the drop; thus fountain currents develop. By adding certain surface tension lowering substances to the medium surrounding drops of oil, chloroform, or mercury, it was possible to obtain changes in form and locomotion of such drops which bore a resemblance to amoeboid movement. (Berthold, Quincke, Bütschli, Rhumbler and others.) Corresponding fountain currents were observed by Rhumbler in certain protozoa, and this author therefore attributed amoeboid movement mainly to changes in surface tension of the protoplasm. It was assumed by these investigators that on the whole the protoplasm is fluid in consistency, although Rhumbler soon recognized that in many cases the peripheral part of the cell is of the consistency of a more or less solid gel. Still he held that even in such cases surface tension changes were the essential factor in amoeboid movement. Later, however, influenced by the observations of other authors, which were not compatible with such relatively simple conceptions, he added to the action of surface tension "gelatinization pressure," by which he meant the pressure upon a fluid exerted by a gelated layer held in elastic tension by this fluid. Although Rhumbler in his theoretical consideration overemphasized surface tension changes as the cause of amoeboid movement, still he made many important observations, and in particular he was the first to describe the transformation of the peripheral hyaline ectoplasm layer into the endoplasm at the posterior end of the cell and the reverse change at the anterior end. The influence of the theory of amoeboid movement and phagocytosis developed by these authors is still potent at the present time and more recent investigators like Tait, Haberlandt and, with certain reservations, also Fenn still uphold these conceptions.

Now, if we had in the case of amoeboid movement merely to deal with changes in surface tension of fluids, this phenomenon, as well as the allied

process of phagocytosis, would hardly have been of interest to colloid chemistry, even if we grant that these changes in surface tension occur in solutions of proteins, which are colloids.

However, there were other observations which, while compatible with the fact that under certain conditions surface tension changes may play a rôle in amoeboid movement, tended to prove that the essential factors underlying amoeboid movement are of a different kind. Thus in 1901 and 1902 we observed in amoebocytes of Limulus that the pseudopods, which were sent out, were not fluid but solid in consistency, as we found, when we touched the amoebocytes with needles and were able to draw out the cells into systems of solid elastic fibers.

We also observed that a pseudopod could be sent out by a cell which was still suspended in fluid; but then it sank down into the fluid medium and on reaching the solid surface of the glass became adherent to the latter. In order to explain the formation of a solid pseudopod of such a character and under such conditions, it was necessary to assume that the essential mechanism underlying pseudopod formation is a change in the colloid state of the protoplasm, which latter in the first phase of the formation of the pseudopod must become more liquid in order to pass into the gel state in the following phase. Jennings soon afterwards, in an extensive study of amoeboid movement in protozoa, carried out experiments which proved that fountain currents may be entirely lacking in amoeboid movement. He surrounded amoebae with india ink particles which stuck to their surface and thus he was able to follow more accurately the changes in the cell during movement. In particular he described a rolling type of movement in certain amoebae possessing a hard pellicle. Subsequently by means of the microdissection apparatus Kite and later Chambers showed the existence of a solid membrane surrounding amoebae. Mast and Root observed that the energy developed by certain amoebae during the formation of pseudopods and phagocytosis was so great that surface tension changes taking place in a fluid protoplasm could not account for this phenomenon. In continuing our studies on amoebocytes we observed modifications in the character of the pseudopods as a result of changes in the medium surrounding the amoebocytes, and beginning in 1919 we analyzed the influence of changes in the medium on the colloid state and consistency of the protoplasm of amoebocytes and the influence of the latter changes on the character of amoeboid movement, agglutination and of factors entering into tissue formation in amoebocytes of Limulus. Similar investigations were undertaken soon afterwards in the case of amoebae by Pantin, Chambers, Mast and Spek, as far as the amoeboid movement is concerned. In this paper we shall attempt to correlate the principal facts established regarding the amoeboid movement in amoebocytes with those found in amoebae, and furthermore, to correlate the changes underlying amoeboid movement with those of phagocytosis, agglutination, tissue formation and thrombosis.*

On the basis of these later observations and experiments, it is thus clear that all these phenomena, related as they are to each other, depend primarily on changes in the colloidal state of various cell constituents, and from this point of view they become problems of the physics and chemistry of colloids. This is so notwithstanding the fact that there exist all possible transitions between the gel and solid state in the protoplasm of these unicellular organisms

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* See papers by Herrera and chambers in this volume. *J. A.*

and that in certain extreme cases amoeboid movement seems actually to depend in the main on surface tension changes of fluid.

THE NORMAL MOVEMENT IN AMOEBOCYTES

Under ordinary conditions, as for instance, in blood serum of Limulus, amoeboid movement is a cyclic process which consists of two phases, namely, (a) the movement of the ectoplasm leading to the formation of the pseudopod, and (b) the movement of the granuloplasm, which is situated at the inside of the ectoplasmic layer. The granuloplasm moves toward and into the pseudopod, thus making possible the movement of the cell as a whole. And in both of these phases again cyclic changes in the consistency and state of contraction of the parts of the cell concerned in the movement occur. As a result of localized softening, accompanied by the movement of fluid from the interior of the cell in the direction toward the anterior pole, the ectoplasm bulges out and forms either an irregularly shaped or a round elevation. The tip of the elevation undergoes a further softening and is drawn out into a finer point which thus rests on a broader base. During this process the ectoplasm apparently hardens again and the completed pseudopod is rigid; the structure thus produced represents a tongue pseudopod of medium width. The central part of the pseudopod is fluid; granules are readily able to move into it without encountering an obstacle within the interior. During this process of pseudopod formation energy is produced and work is accomplished; the momentum of the extending pseudopod at this time is so great that it is able to push out into the surrounding medium, overcoming resistance within the latter and its own gravity. This development of energy must depend on changes accompanying or preceding the alterations in the colloidal state of the protoplasm, on which the pseudopod formation depends. After the ectoplasm has come to a temporary rest, following the completion of the pseudopod, the granuloplasm, and especially the denser layer of hyaloplasm around it, soften sufficiently—presumably through taking up of fluid—to allow the movement of the granuloplasm into the pseudopod. This movement under normal conditions takes place slowly and connectedly. It begins at the anterior pole and extends from here, as far as one can determine, in a wavelike motion toward the posterior pole. At the posterior pole the granuloplasm is often inactive, and in typical cases a furrow separates this inactive posterior from the active anterior part of the granuloplasm. Thus a knob is demarcated here from the rest of the cell. The ectoplasm of the posterior pole contracts as a whole, apparently giving way to an elastic tension, and thus carrying along with it the inactive knob. In this way the cell as a whole moves. However, before the granuloplasm in its slow movement has reached the tip of the pseudopod, the latter begins to send out a new pseudopod which is again the result of a localized softening of the ectoplasm; thus a new cycle begins. The pole of the amoebocyte remains approximately constant for a number of these cycles, but certain variations may appear.

In addition to these typical movements other movements may occur. There may, for instance, take place a simultaneous softening of the ectoplasm and the adjoining granuloplasm in certain places, thus leading to *pari passu* movement. When the latter affects the furrow which demarcates the knob, it may cause an obliteration of this furrow and with it of the knob.

The Mechanism of the Movement of the Granuloplasm

As to the mechanism by means of which this movement of the granuloplasm is carried out, it is probably the same that is active in the case of the ectoplasm, namely, the softening of certain parts of the intergranular hyaloplasm, presumably associated with or caused by the taking up of fluid. Through this movement of the anterior part of the granuloplasm a pull is exerted on the posterior part and thus in addition elastic forces are set free. Subsequently the more posteriorly situated parts of the granuloplasm soften and move forward and the stiff posterior end of the cell contracts apparently under the influence of elastic forces. The intergranular hyaloplasm is continuous with and is essentially the same material as the ectoplasm. The same forces are therefore active in both. After the granuloplasm has reached a certain level in the pseudopod, it stiffens again and its movement comes temporarily to a standstill.

Plasticity of the Amoebocyte and Relation between Consistency of the Cell and Character of Ectoplasmic and Granuloplasmic Movements

The amoebocyte represents a very plastic organism as far as change in the character of the amoeboid movement and secondary transformations in the cell, or in aggregates of cells (amoebocyte tissue) in response to environmental conditions, are concerned. The tongue pseudopodia of medium width, which we have considered so far, represent an intermediate condition, from which all possible transitional states lead, on the one hand, to the formation of thread pseudopodia and, on the other hand, to the formation of balloon pseudopodia. Those factors which increase the consistency of the protoplasm and cause the cells to be very contracted and hard and their outlines sharp and round, lead also usually to the production of very fine threadlike pseudopodia, which in some cases may show dichotomous ramifications. At the same time they result in the withdrawal of fluid from the cells and tend to diminish the size of the latter. On the other hand, all those conditions which render protoplasm soft, flabby and the cell broad and flat, tend to cause the production of balloon pseudopodia. These factors lead to the taking up of outside fluid into the cells. Consonant with the character of the pseudopods in these various cases, is the character of the granuloplasm. It is hard and viscous, moving slowly and with difficulty in the first case, while in the second case it tends to be of a relatively low viscosity, flowing readily, occasionally even pouring into the pseudopod. In the latter condition the cells, on account of the fluid which they have taken up from the outside, are usually larger; their granules may also be somewhat swollen and more at a distance from each other. In extreme cases the intergranular hyaloplasm may become so fluid that the granules may carry out Brownian movements within the cell.

Thread Pseudopodia, Balloons and Circus Movement

According to gradations in the osmotic pressure of the surrounding medium we can obtain all possible gradations in the character of pseudopodia from thread pseudopodia in hypertonic, to very broad tongue and balloon pseudopodia in certain hypotonic media. Thread pseudopodia may also be sent out by cells which have migrated from pieces of amoebocyte tissue which has been for some time previously kept in a medium of a certain acidity. There is

always a correspondence between the consistency of the protoplasm of the cell caused by the media, and the character of the pseudopodia. Because the consistency of the granuloplasm and ectoplasm is so great in the case of the formation of thread pseudopods, the latter usually remain unchanged and persist for a long period of time, while new ones may appear in different parts of the periphery of the amoebocytes at a time when the old ones are still visible. Thus we often observe under these conditions a multiplicity of thread pseudopods, and a lack of polarization and of locomotion on the part of the amoebocytes. We can also produce multiple thread pseudopodia in cells which have migrated out from the piece of amoebocyte tissue, by pouring on the cover glass, to which the cells are attached, a slightly hypertonic solution of ammonium carbonate.

In the case of balloon formation we find a series of gradations in the decrease in consistency of the protoplasm. On the one hand the ectoplasmic layer lining the balloon may still be relatively firm and change secondarily into a tongue pseudopod. Next we find a polarization of balloons, the latter developing at a definite pole of the cell; in this case the granuloplasm still moves into the balloon relatively slowly and connectedly, but usually reaches the tip of the balloon and a new balloon may form on top of the first one. Thus the cell is able to move as a whole. In case of a more pronounced decrease in consistency, the polarity is lost, balloons frequently form in relatively rapid succession in various parts of the cell circumference and the granuloplasm pours in rapidly; sometimes even granuloplasm and ectoplasm may bulge out simultaneously owing to a pronounced fluidity of the protoplasm in a certain place, thus leading to *pari passu* movement. Under these conditions the cell has lost its power to move as a whole, the movement in one direction being soon neutralized by a movement in another direction. We may then conclude that a medium consistency of the protoplasm is necessary for effective locomotion of the cell. Too great or too low a consistency leads to a loss of polarity and of the power of locomotion. The excess fluid, to which is due the greater fluidity of the protoplasm in the case of balloon formation, is derived from the surrounding medium; but this fluid, after it has once been taken up by the cell, moves from one part of the cell to another, wherever a balloon is in process of development. Under other conditions also we may observe that the outside fluid can directly enter the cell; this is especially the case at the anterior pole where pseudopods are found; they may then take up fluid, swell and thus become transformed into balloons. It is evidently at the anterior pole that the metabolic changes take place with the greatest intensity.

Balloon formation occurs in hypotonic media, and also under the influence of certain ions which have a softening influence, as for instance, in solutions of KCl, especially if they are hypotonic. Furthermore if cells have been kept for a long period of time in the same medium of serum, the amoebocyte tends to take up fluid, to become flabby and to form balloons. We may assume that the cell metabolism in some way becomes abnormal under those conditions.

If the fluidity of the ectoplasm and granuloplasm becomes very marked, only a thin film of ectoplasm may surround the balloon and the latter may flow as a drop around the cell circumference, the granuloplasm pouring into the flowing drop, and thus moving in a circle around the remnant of the amoebocyte. Thus circus movement is produced. Following a period of active circus movement the cell may round off again, after the film, separating the

balloon from the periphery of the cell, has become dissolved; the granuloplasm stiffens somewhat and the cell thus comes temporarily to a state of rest, which lasts until a new period of circus movement begins. In principle we have, in case of circus movement, apparently to deal with the same processes as in ordinary amoeboid movements. Certain localized metabolic changes in the cell lead to softening processes in the ectoplasm; this condition is followed by a softening in the granuloplasm which thus pours into the balloon. In this case also the change seems to be propagated from the granuloplasm nearest the balloon to parts more distantly situated. Such a period of activity is followed by a process of hardening, contraction and rounding off, which lasts up to the beginning of the new period of activity. However, the circus movement differs from other types of amoeboid movements in that it occurs in cells in which the hyaloplasm and also the granules have previously taken up much fluid under the influence of marked hypotonicity of the surrounding medium, especially in cases in which the hypotonic solution contains KCl. Yet the latter substance may produce circus movements occasionally even in isotonic media, if it is present in these in suitable sufficiently weak concentrations. The addition of somewhat larger amounts of potassium on the other hand again stiffens the protoplasm and thus prevents the circus movement.

SNAIL MOVEMENT

An interesting modification of amoeboid movement, which throws light on certain underlying mechanisms which play a part in this process, is found under certain conditions in which a sharp tongue pseudopod is sent out in the usual way through softening and subsequent hardening of the ectoplasm, while the granuloplasm has become so stiff that it is unable to undergo the rhythmic changes which lead under usual conditions to its movement. Instead, the contraction of the extended pseudopod pulls the cell along just as the contraction of the extended foot of the snail leads to the forward movement of its house. Such an effect may be observed in relatively strong solutions of alkali (m/60-m/100 NaOH) in m/2 NaCl, or in isotonic or only weakly hypotonic solutions of KCl, but occasionally also in other media. We must assume that after the completion of the process of extension and during the process of hardening the extended ectoplasm gains elastic properties and retracts somewhat in the same manner as a stretched rubberband. It is probable that similar processes take place normally in the ectoplasm and granuloplasm during amoeboid movement. We assume that the process underlying this retraction is similar in character to the retraction which takes place in Limulus blood following its pseudo-coagulation, where likewise the stretched cell fibers gradually retract, or in true fibrin in the blood of other animals. In this case during the process of conversion of fibrinogen into fibrin a polarized arrangement of the micellae or other changes must occur as a result of which the fibers gain elastic properties and retract as if they were in a stretched condition.

THE MECHANISM OF EXTENSION

In normal amoeboid movement the amoebocyte in the beginning of the cycle is in a state of contraction; temporarily a moderate extension occurs as a result of the sending out of the pseudopod and the movement of the granuloplasm into the pseudopod, but the cycle ends with the contraction of the posterior parts of the cell, so that at the conclusion of the cycle the cell has returned to its contracted state. In many cases, however, the constitution of

the protoplasm becomes changed in such a manner that it is no longer able to contract properly at the posterior end after the earlier phases in the cycle have been finished. We have then to deal with inner causes leading to a more complete extension of longer duration. Under these conditions extension is a pathological type of amoeboid movement, the result of changes within the protoplasm of the cell. Such an alteration may be found in a cell which becomes broader and softer, as the result of the taking up of fluid into the protoplasm from the surrounding medium. We observe it therefore especially in cases in which the amoebocyte has the tendency to form broad tongue pseudopodia or balloons; the granuloplasm then may move into the pseudopod or balloon, but the cell subsequently does not contract. Media which thus soften the protoplasm and make it flabby are, for instance, hypotonic solutions, certain solutions of potassium salts, and in general also nitrates; calcium salts as well lead to an extension. Isotonic solutions of alkali have a similar effect except in relatively strong concentration, which on the contrary may lead to a state of contraction. On the other hand, sodium, SO_4, and acids tend to cause a hardening and contraction of the cells. As stated, relatively strong alkali in isotonic NaCl solutions also causes a contraction of the cell. We must make allowance, however, for the fact that if we study amoebocytes by the method of tissue culture, in which a piece of amoebocyte tissue is surrounded by a certain medium for a relatively long period of time, substances may be extracted from the tissue, especially as the result of disintegration of a certain number of cells, which may alter the constitution of the medium in such a way that it becomes somewhat similar to Limulus serum and its effects are then intermediate between those exerted by Limulus serum and by the specific solution to be tested. Thus NaCl can under these conditions allow a certain relaxation of the amoebocytes, while without this admixture it causes a very marked contraction of the amoebocytes. In general, cells surrounded by a certain medium for a longer period tend to take up fluid in the course of time and to become more and more flabby, provided the character of the medium does not counteract such a tendency. Thus the cells gradually extend in Limulus serum, as they do likewise in NaCl solutions, to which some serum has been admixed; also in isotonic solutions of alkali, of medium concentration, such an extension gradually takes place.

This extension, being the indication of an abnormal softness of the cell, usually makes the cell very sensitive to all kinds of injurious effects. It is therefore the extended cells which dissolve first, if we change the medium surrounding the tissue. In addition to the decreased resistance of amoebocytes in this state of extension, there is in such cells a greater surface exposed to the action of the solutions. Thus it comes about that in many cases extension is the precursor of the degeneration of the cells; but under certain conditions, especially if the extension is still moderate, stimulation of various kinds may again cause a contraction of the cells; even cells which are not only extended, but which have undergone a hyaline degeneration, may again contract temporarily and send out pseudopodia. Extension depends thus in these cases upon processes which are to a certain extent reversible.

STEREOTROPISM IN RELATION TO AMOEBOID MOVEMENT AND EXTENSION OF TISSUE CELLS

Extension as well as efficient amoeboid movement leading to actual locomotion takes place only in contact with solid surfaces. In both these processes

the cell is adherent to the surface on which it is lying; this adhesion can occur only if the outer film, at least, of the solid ectoplasm takes up a sufficient amount of fluid. In the case of extension, it is self-evident that contact with a surface of a certain density is necessary for this process. In the case of amoeboid movement the amoebocyte is able to send out a pseudopod into the surrounding fluid, but actual locomotion again occurs only in contact with a solid surface. If a pseudopod is sent out into a fluid medium, it sinks down into the fluid and a part of the granuloplasm may move into the base of such a pseudopod; but at the same time the cell body remains fixed to the surface. Effective locomotion occurs if also the pseudopod can attach itself to a solid surface; only under this condition can the pseudopod and the posterior part of the cell contract in such a way that the cell as a whole moves. It can be shown that the tissue cells of higher organisms likewise move effectively or extend only in contact with solid surfaces; this mode of reaction has been designated by us as tissue stereotropism. We have every reason to assume that the mechanism which determines the necessity of contact with a solid surface in the case of amoeboid movement and extension of amoebocytes holds good in the case of tissue cells as well, and in particular we may conclude that also in the case of tissue cells undergoing these changes we have to deal not with drops of fluid spreading out on a surface as a result of surface tension changes during the locomotion and extension of these cells, but with rhythmic changes in consistency, similar to those which we described in amoebocytes, occurring in an originally solid ectoplasmic layer and in that part of the cell which corresponds to the granuloplasm of amoebocytes.

Agglutination of Amoebocytes and Formation of Amoebocyte Tissue

The same characteristic of the amoebocyte which causes its agglutination to solid surfaces and which is also responsible for the drawing out of the posterior end of the cell into threads, if in the course of amoeboid movement it is pulled away from places to which it agglutinates, causes agglutination of amoebocytes to each other. Under normal conditions the amoebocyte floating in the blood channels of the animal is not sticky; it is an elliptic, flat disc, transparent, with the nucleus in the center and small granules situated underneath the smooth ectoplasmic layer. If blood is withdrawn through an incision into the joint of a Limulus and the blood is allowed to flow into a vessel without special precautions, as the result of the changed environmental conditions and especially of the mechanical irritation, the amoebocytes round off, send out pseudopodia and stick to each other. During this process many cells are drawn out into long threads and within a short time the blood appears as a solid mass resembling clotted blood. In reality we have to deal with a pseudocoagulation, consisting in an agglutination of the blood cells. During this process the protoplasm of the amoebocytes probably takes up fluid, softens, extends and subsequently hardens, at the same time gaining elastic properties, in consequence of which it retracts. Thus the physical characteristics of the material produced are similar to those of true fibrin, which also possesses elastic properties and retracts after the fibrinogen, during its conversion into fibrin, has been drawn out into solid fibers. In both cases work is accomplished during this retraction and interfibrillar fluid is squeezed out.

If in collecting blood we use certain precautions the blood may be obtained in a condition intermediate between the state of pseudocoagulation and the state in which the cells are nonagglutinated. In this case the cells sink down

to the bottom of the vessel, sticking to each other more or less tightly. The sending out of pseudopodia, cellular disintegration and the subsequent retraction of the cellular material are much diminished under these conditions. In this manner a layer of amoebocytes is obtained, which resembles ordinary tissue of higher organisms in some essential respects and can be treated as such. If portions are excised in this layer and thus wounds are made, the cells near the wound become active, migrate into the defect, and a process comparable to wound healing occurs. Such small portions cut out from the piece can be used for tissue culture experiments. The cells move out from the periphery of the piece in a centrifugal direction into the surrounding medium. The agglutination of the amoebocytes, which leads to the formation of this layer of tissue, depends upon a change in the colloidal state of the ectoplasm, which apparently takes up just enough fluid to make it sticky. However, under various conditions this stickiness, dependent upon the consistency of the outer layer, differs very much. All those factors, which cause a hardening of the protoplasm, diminish the agglutination; at the same time they increase also the state of contraction of the cells, they produce a greater sharpness of the pseudopods, raise the viscosity of the granuloplasm, cause the granules to be small and tightly packed together, and slow the movement of the granuloplasm and of the cell as a whole. Those factors, which produce a softening of the protoplasm and result in a taking up of fluid on the part of the cells, act in every respect in the opposite way; they favor extension and increase the agglutination of the cells. Among the factors having the first named effects are relatively strong acids in isotonic solutions, temporarily also relatively strong alkalies in isotonic NaCl solutions, hypertonicity, SO_4, Na ions, cold; the second kind of effects are exerted by hypotonic solutions, within certain limits of hypotonicity, by K, NH_4, and NO_3 ions within certain concentrations, by blood serum of Limulus, alkali solutions, except in relatively strong concentrations, and by a higher temperature which must not exceed a certain limit. These statements hold good under conditions such as obtain in tissue culture experiments, when the amoebocyte tissue is exposed to the action of the medium for a longer period of time and when the medium can extract certain constituents, mainly of a protein character from the amoebocyte tissue.

Not only do certain agents as, for instance, acids of sufficient concentration, diminish the agglutination between blood cells, which in certain other media would agglutinate with each other whenever they meet in the course of their migration, but they can even cause a loss of agglutination, a desagglutination, between the cells which have joined together in the tissue layer. Thus in acid solution of suitable concentration agglutinated cells may detach themselven from each other, as well as from the surface on which they rest, and in large numbers sink down in the fluid until they are held back by the surface film of the latter.

In addition to this desagglutination, occurring only in certain media which have a hardening effect on the ectoplasm of the amoebocytes, a reaction against the state of agglutination sets in in agglutinated cells provided they stick to each other only on one side, while the other part of the circumference is surrounded by a fluid medium. Amoeboid activity soon begins in one or the other of two agglutinated cells in such a way that a free part of the cell periphery becomes converted into an anterior pole and develops a pseudopod; thus the amoebocyte tends to move away from the cell to which it adheres.

There is furthermore a tendency towards continuity in the location of the active pole and the cell usually moves for some time in the same direction, until it meets another cell to which it sticks. Inasmuch as the chances of meeting other cells are the greater the nearer the cells are to the piece of tissue from which they originally migrated, the cells would naturally tend gradually to assume a centrifugal direction in their movement. Thus it is possible to explain the centrifugal growth which we observe in tissue cultures.

It is primarily a change in the environment of amoebocytes which causes that alteration in the consistency of the ectoplasm which underlies pseudopod formation, as well as agglutination. Agglutination is the basic factor in tissue formation. Now it is possible to produce a corresponding change of environment within the living Limulus by introducing a foreign body into the blood channels of the animal. In contact with the surface of such a foreign body, which differs in its character from that of the wall of the blood channels, the amoebocytes undergo similar changes to those occurring outside the body and in consequence of these changes agglutination of the amoebocytes around the foreign body takes place, leading to the formation of a structure which corresponds to a thrombus. Thus the phylogenetically oldest thrombus formation is related to tissue formation and underlying both these processes is the change in the character of the ectoplasmic cell layer which leads to agglutination.*

DEGENERATIVE CHANGES IN AMOEBOCYTES AND AMOEBOCYTE TISSUE

In amoebocytes which have grown out from pieces of amoebocyte tissue in certain media, secondary degenerations take place in the course of time; nonreversible injuries gradually occur and their effects accumulate. If we take as standard the contracted granular cell undergoing amoeboid movement in the way we have described, these secondary changes are of a pathological character; but, on the other hand, if we take as standard the rigid, non-agglutinative and only passively movable disc as we find it in the normal blood channels, then the processes of agglutination and amoeboid movement themselves must be considered as pathological reactions. These secondary degenerative changes are a further development and an accentuation of the modifications in consistency of the protoplasm and in the amoeboid movement which we have already discussed, and which are produced essentially by abnormal conditions prevailing outside the body. We can classify these changes according to the effect which various environmental factors have on the consistency of the protoplasm of amoebocytes.

a. Degeneration of amoebocytes in hardening media such as hypertonic and even isotonic solutions of NaCl and Na₂SO₄. Here the cells are contracted; they send out sharp pseudopodia and show only a very slight tendency to extend. Gradually the amoebocytes lose here their granules and undergo hyalinization. Even these hyalinized contracted cells may still continue to send out sharp and often even multiple thread pseudopodia and they also may show restricted amoeboid movement. In addition to hyalinization further degenerations take place in which the cell flattens out, loses its granules and, consists of a more hyaline resistant outer framework and an inner vacuolar zone, in which solution has taken place. This change may be designated as cytolysis.

* See paper by H. A. Abramson in this volume. *J. A.*

b. Degeneration in Limulus serum, in deproteinized serum, sea water or similar solutions. In Limulus serum the cells gradually extend, lose their granules, become transformed into very much spread out hyaline plates and at last some of them may dissolve. In deproteinized serum and similar media the same change may take place, but in addition cytolysis and hyalinization and complete disintegration may occur. Calcium and to some extent also magnesium salts have the tendency to produce gradually a broadening and flattening out of the cells, a loss of granules and finally a very pronounced cytolysis, in which only an outer hyaline refringent framework of the cell is left. In the end even the latter may become dissolved.

c. Degeneration in softening media, such as hypotonic solutions in general, and in particular in mixtures of salts and nonelectrolytes, in hypotonic potassium chloride solutions, and in hypotonic solutions of weak alkali. In addition to a very marked extension of the cells, solution of granules and subsequent solution of the whole cell and to cytolysis, which we also encountered under other conditions previously mentioned, there may be an accentuation of those changes in the amoebocytes which underlie the formation of balloons. The increasing amount of fluid entering the cell may lead to a widening of the balloons into crescents and courts and in the end structures resembling fertilization membranes of fertilized ova may develop. All these formations may be considered as abnormal pseudopodia. When the last named changes have occurred the cells thus affected have usually lost their normal reactivity; their granuloplasm has become stiff and such amoebocytes may be considered as dead. In other cases, as for instance, in certain mixtures of salts and nonelectrolytes, a triangular or quadrangular framework, enclosing a fluid zone and a central remnant of granuloplasm, may be produced. A further going change may be observed in media which make the cell protoplasm soft and sticky, such as hypotonic solutions of weak alkali and certain solutions of non-electrolytes; here the cells may be readily converted into radially arranged fibers in which, in the center, the drawn out, rod-like nucleus may still be visible. Or, if this softening effect becomes general, whole systems of long, radiating fibers, extending far into the surrounding medium and without any trace of cell structure, can develop. In other cases of pronounced injury the agglutinated cells composing the margin of the piece may extend, lose their granules and form a layer of regular hyaline cells not unlike a layer of epithelial tissue. Especially certain solutions containing glycerin are well suited to bring out this transformation.

DEGENERATION AND TISSUE FORMATION

The changes which we have described in the preceding chapter are in principle the main types of degeneration that occur in amoebocyte tissue; they are of interest from two points of view. In the first place the character of these secondary changes gives further insight into the consistency and structure of the cell, and secondly, there exists a certain relation between these degenerative changes and processes which occur during tissue formation. Primarily through agglutination the cells are joined into tissues; secondarily, certain paraplastic structures are produced from cell substance or from material given off by cells through processes which are akin to certain degenerations, in particular to changes as we observed them in amoebocyte tissue under unfavorable environmental conditions. Thus, for instance, fibers develop from cell material and perhaps fibers traversing cells and uniting neighboring

cells may also develop. Extension of cells in contact with solid substances is a further factor of importance in tissue development and differentiation. In the amoebocyte tissue formations closely resembling tissuelike structures may develop in the outgrowing cells as the result of these secondary changes; in particular also under certain conditions formations resembling epithelium may be observed. Of course we have in these cases to deal not with identity between the results of secondary changes of a degenerative character in amoebocytes and normal tissues, but some essential factors underlying both are undoubtedly present. Injurious conditions leading to degenerative processes are also of primary importance in the differentiation in normal tissues. This is, for instance, quite definite in the case of the epidermis where the production of specific paraplastic structures is initiated or intensified as the result of increasing distance from the source of oxygen supply. It is also noticeable in connective tissue, where fiber formulation becomes the more pronounced the less favorable the conditions of nourishment and the less efficient the vascularization. In all these circumstances paraplastic structures are produced in increasing quantity, as we observed them in amoebocyte tissue under injurious conditions leading to degeneration.

PSEUDOPOD AND FERTILIZATION MEMBRANE

We have seen that in amoebocytes under certain conditions structures develop which resemble fertilization membranes of ova. Apparently we have to deal here merely with a superficial analogy between two kinds of formations developing in totally different cells, under different conditions and leading to different results. However, notwithstanding these differences, some essential similarities do exist. In both cases a fluid zone develops in the peripheral part of the cell and separates at this point a solid membrane from the rest of the cell. This membrane may be either preformed (in ova and perhaps in amoebae) or it may represent a condensation product of the outer hyaloplasm (amoebocytes). In both cases a stimulus leads to those changes which attract fluid and death of the cell follows if the intensity of this process is increased. In the amoebocytes the pseudofertilization membrane represents already a pathological extension of the process leading to pseudopod formation and is followed by death. In both cases the production of the liquid peripheral zone is the initial stage which is followed by further cyclic changes succeeding each other in a definite sequence. From this point of view, in order to express these basic similarities, we may characterize the pseudopod as a localized fertilization membrane and the fertilization membrane as a generalized pseudopod.*

INTERNAL FACTORS IN AMOEBOID MOVEMENT AND IN CELL DEGENERATION

While certain conditions in the surrounding medium are essential in initiating amoeboid movement and in determining the character and intensity of the degenerative changes which may take place in the amoebocyte tissue, they are not the only variables that influence the result. There are in addition internal factors, located within the cells, which coöperate in these processes. We may distinguish two sets of such internal factors. In the first place the tissues obtained from different Limuli differ in many cases as far as the softness or hardness of the amoebocytes and the tendency of the latter to extend or contract and to form sharp or round pseudopodia is concerned. In the

* See paper by E. E. Just in this volume. *J. A.*

case of some tissues degeneration sets in very rapidly in cells leaving the tissue; the amoebocytes appear flabby and their pseudopods are roundish, the cells soon extend and readily disintegrate. Other tissues resist the effects of injurious solutions much more effectively; with the aid of substances extracted from the tissue the amoebocytes may grow out well even in isotonic NaCl solutions. There is a second factor that in some instances may influence the power of resistance of amoebocyte tissue to injurious conditions, namely, the time that has passed since the piece of tissue was placed in the artificial medium. With increasing time the cells may become less resistant; they seem gradually to take up some fluid and their tendency to the formation of balloons, instead of sharp tongue pseudopodia increases; they also degenerate more readily with increasing age of the cultures.

THE PROTECTIVE EFFECT OF ACID AND ALKALI ON AMOEBOCYTE TISSUE IN TISSUE CULTURE

As we have seen, cells grow out from pieces of amoebocyte tissue surrounded by various salt solutions; they then undergo sooner or later degenerative changes, the time when these occur depending upon the character of the surrounding solution and of the tissue used. In very many cases the onset of degenerative changes can be much retarded by the addition of suitable concentrations of acid and to a less extent also of alkali to isotonic, and likewise to hypertonic and hypotonic solutions of sodium chloride. Both acid and alkali if added to the amoebocyte tissue in relatively strong solutions cause at first a contraction of the amoebocytes which thus become round and oval, and either do not send out any pseudopodia, or send out only very short and sharp ones. During this phase of rigidity the outgrowth from the piece of tissue is inhibited. Gradually the pH in the solution to which the acid or alkali has been added alters in both cases in the direction towards the neutral point owing to the action of substances extracted from the amoebocyte tissue, and as the result of this change in the medium the rigidity of the cells diminishes, they regain their plasticity, send out good tongue pseudopodia, form knobs and some of them begin to extend and may even dissolve. At the same time, after addition of suitable quantities of acid and alkali to the medium, the outgrowth and relative preservation of the cells usually surpass greatly those observed in the approximately neutral control solutions of sodium chloride. If the outgrowth of the acid or alkali is still further diminished, the condition of the cells approaches those obtaining in the controls; very weak alkali solutions may increase the softness of the cells and thus the degeneration in the latter media may be even more pronounced than in neutral NaCl solutions.

An analysis of the effects of acid and alkali observed under these conditions shows them to be complex, but it may prove to be of interest on account of a certain similarity between the conditions prevailing in these tissue cultures and in the living organism. At least three sets of factors come here into play: (1) Relatively strong solutions of acid and alkali cause a contraction of the amoebocytes and either restrict the formation of pseudopods or tend to make them sharp. The cells are round or oval and possess sharp outlines. In acid the granules are small and tightly joined to each other. (2) The continued action of acid hardens the cells and makes them more resistant to various injurious influences. (3) Acid and alkali extract protective substances, especially protein, from the amoebocyte tissue and these

substances added to the pure salt solutions greatly mitigate the injurious effect of he latter on the amoebocytes. If we extract amoebocyte tissue with acid in isotonic NaCl solution, a culture medium may be obtained which, as far as its beneficial action is concerned, surpasses even the blood serum of Limulus, and this beneficial effect may be observed also in cases in which the extracted substances have completely neutralized the acid used for extraction.

PROTECTIVE EFFECT OF PROTEINS ON AMOEBOCYTE TISSUE. PERMEABILITY OF AMOEBOCYTES

The presence of proteins in the medium surrounding the amoebocyte tissue is probably necessary for the preservation and continued amoeboid movement of the amoebocytes. We have already referred to the beneficial effects on these cells of extracts of amoebocyte issue made with acid and alkali solutions. On the other hand, deproteinized Limulus serum is apparently more injurious for the amoebocytes than isotonic NaCl solution. The amoebocytes behave therefore in this respect very differently from amoebae which are adapted to media free of protein; thus sea water amoebae behave normally in sea water. We may perhaps have to distinguish between the reactions and needs of cells adapted to a protein-containing environment, as it exists within the body, and of others, adapted to a life outside the organism.

There is possibly a relation between the need for protein on the part of the amoebocytes and the readiness with which certain inorganic constituents penetrate into the interior of these cells. Thus weak concentrations of acid and alkali can be shown to enter these cells rapidly without killing them. In the case of certain salts, like KCl, it is likewise probable that potassium produces its effects after having entered the cells. The specificity of these effects suggest this conclusion; the same may possibly hold good in the case of other salts.

MEMBRANE FORMATION IN AMOEBOCYTES

When under certain conditions, especially of a pathological character, we observe the actual formation of a membrane in the periphery of the amoebocytes, this must be due to secondary transformations of the ectoplasmic hyaloplasm. Normally the elevation of a membrane-like substance occurs at the anterior pole during the pseudopod formation. But even here we may have to deal, as we stated above, primarily with the swelling and flowing out of the ectoplasm, to which is added perhaps some intergranular hyaloplasm and not with the elevation of a preformed membrane. At the same time the central fluid fills the core of the pseudopod. If we consider that under pathological conditions the cell as a whole takes up more fluid, and a widening and rounding off of the pseudopod takes place so that in the end it may surround the whole cell and that at the same time in proportion to the increased amount of fluid taken up by the cell and the resulting more fluid consistency of its protoplasm, a thinning out of the ectoplasmic layer of the pseudopod takes place, so that it becomes a thin film raised up by a relatively large amount of fluid, which separates it from the remaining granuloplasm, then we can understand the formation of these membrane-like structures through secondary condensation processes of the ectoplasm (hyaloplasm) without having to assume the existence of a preformed distinct membrane covering the ectoplasmic layer.

A Comparison between the Structure and Amoeboid Movement of Amoebae and Amoebocytes

It will be of interest to compare with our findings on amoebocytes the observations of various authors on amoebae, in order to determine what are the common underlying factors in amoeboid movement. We shall find some essential similarities but also some differences in these two classes of organisms. This can be readily understood if we consider the differences in the media in which both kinds of cells normally live. The amoebae as far as they have served for the analysis of amoeboid movement are free living organisms, adapted to sea or fresh water, where they are exposed to considerable alterations in temperature, and to the mechanical action of an environment which is quite variable in its character. Also the osmotic pressure and the hydrogen ion concentraion of the surrounding fluids are in the case of amoebae subjected to greater variations than in the case of the sheltered amoebocytes, living in smooth channels within the animal body, in a protein-containing medium. Furthermore the amoebae are exposed to irregular motions of the medium, while the medium surrounding the amoebocytes is in regular rhythmic motion. Amoebae must divide, while in the case of amoebocytes the propagation occurs in tissues from which a new supply of cells moves into the blood. The amoebae have therefore a structure which enables them to resist conditions to which the amoebocytes would succumb. The former are surrounded by a distinct, firm, elastic membrane, inside of which is a viscous layer of ectoplasm. According to the recent studies of Mast in amoeba proteus, there is found between the outer membrane (plasmalemma) and the more solid ectoplasm (plasmegel), which latter has an alveolar structure, a hyaline fluid zone. The central endoplasmic material (plasmasol) is a suspension with a viscosity approaching that of water. In amoebae the outer layer is thus in general represented by a distinct membrane of much greater firmness than the ectoplasmic layer surrounding amoebocytes. In amoebae a suitable medium can be prepared consisting of a mixture of inorganic salts in balanced solutions, while in the case of the amoebocytes such a balanced salt solution does not exist; they need the protection of certain proteins and, in this respect, resemble tissue cells of higher organisms. In consequence of their structure, amoebae in general are more rigid in their response to changes in environment than amoebocytes, which are extremely plastic. In amoebocytes, phenomena which demonstrate their relationship to tissue cells, like agglutination and the production of tissue-like and paraplastic structures, are very prominent, while these are lacking in the more stable amoebae.

As far as the character of amoeboid movement is concerned, we find considerable differences in different types of amoebae and some variations even within the same type. More recently Pantin found in amoebae of the Limax type in the beginning of amoeboid movement a softening in the ectoplasm at the anterior end which is followed by a streaming of the relatively liquid endoplasm in the center of the cell in a forward direction. Having reached the anterior pole, it turns sidewise and gelates in contact with the ectoplasm. Thus the fluid endoplasm becomes transformed into solid ectoplasm near the anterior pole, while at the posterior pole the opposite process takes place, the solid ectoplasm becoming converted into liquid endoplasm and streaming forward. The ecto-endoplasm process of Rhumbler thus occurs in the amoeboid

movement of these organisms. In certain respects the cell grows therefore through apposition; it is built up at the anterior and torn down at the posterior end. Chambers also had previously observed the lateral flowing back of liquid material and its subsequent changing into a jelly. In Pelomyxa there are according to Spek two kinds of movement: (1) The fountain-streaming, corresponding to the original description of amoeboid movement given by Rhumbler, and indicative of a liquid state of the whole protoplasm, with the exception of the membrane. (2) Under certain conditions there may develop in Pelomyxa an outer layer which is clear and hard and from which multiple pseudopodia are sent out. Similarly in amoeba polypodia several types of movement occur. (Spek.) Either multiple, long and threadlike pseudopodia are sent out from a central round granular body, or, in other cases, the whole mass of the amoeba may flow along assuming the shape of a leaf, or it may send out pseudopodia and thus change its shape. The former process bears some resemblance to the formation of thread pseudopodia and the flowing of the whole amoeba corresponds to *pari passu* movement in amoebocytes. Thread pseudopodia are also produced in Astrorhiza. Here they are sticky, can contract and thus cause locomotion, not unlike the snail movement which, in certain media, may occur in amoebocytes. Dellinger also has described the contraction of pseudopods after the distal ends of the latter have attached themselves to the surface on which they move. In amoeba terricola the consistency of the cell is so great that only small folds are produced on the surface; but in another related type, which is less viscous, Spek observed the formation of broad balloon-like pseudopods, into which the endoplasm moves gradually and without reaching its tip. In this case a more consistent layer seems to surround the endoplasm and to separate it from the hyaline material of the pseudopod. Different again is the rolling movement which Jennings observed in certain cases; in this type of movement the lower surface of the cell is more solid and fixed to the base on which it rests, while the more liquid upper and anterior portions roll forward. In addition to these types of amoeboid movement Rhumbler has described certain modifications which correspond exactly to changes experimentally produced in amoebocytes. Thus the multiple thread pseudopodia of amoeba radiosa are not unlike those produced in amoebocytes in hypertonic solutions of NaCl and in Na_2SO_4. In amoeba Limicola there occurs the circus movement of amoebocytes which we found in dilute and hypotonic solution of KCl, while in amoeba blattae the drop pseudopodia of amoebocytes have been observed. In comparing the corresponding activities in amoebae and in amoebocytes we may conclude: (1) The same end is accomplished in amoebae and in amoebocytes by different means, which, however, in general have in common certain changes in the colloidal state of cell constituents as the principal factor underlying this activity. (2) The differences which have been observed in different types of organisms or within the same type at different times depend principally upon differences in the colloidal state of various constituents of the cell. (3) A great variety of changes found spontaneously in different types of amoebae can be produced experimentally in amoebocytes, and the changes thus produced in the latter cells again depend on corresponding changes in the colloidal state of the protoplasm. (4) The ability of the amoebocytes to undergo modifications in their mode of pseudopodial activity and movement is much greater than has been observed in the case of amoebae, because the structure of the former is much less rigid and their protoplasm more readily undergoes

alterations in response to environmental conditions than is the case in amoebae, where in general the modifiability is much more limited.

As to the effects of acid and alkali, the observations which we made in cultures of amoebocyte tissue cannot apparently be duplicated in the case of amoebae. This may be expected if we consider the differences in conditions prevailing in the tissue culture and in experiments in which we subject individual cells directly and suddenly to the influence of a change in the character of the medium. Even in the case of amoebocytes the results obtained in using these two methods are not identical. In experiments carried out with the tissue culture method we found an essential parallelism in the action of acid and alkali; both produced a series of sequences of a similar character in accordance with the initial strength of the acid and alkali used and with the gradual changes in the pH of the medium which occurred subsequently. Yet there was at the same time noticeable a greater tendency on the part of acid to cause a hardening and, on the part of alkali to cause a softening, extension and solution of the cells, especially in sufficiently low concentrations. Our more recent observations concerning these effects agree with our earlier experiments in which we found a parallel action of acid and alkali on the preservation of the amoebocytes and especially of their granules. In amoebae the essential effect of acid observed is a hardening and coagulation of the protoplasm, while in the case of alkali a softening or liquefying effect takes place.

If we compare the action of salts and ions on amoebocytes with the corresponding action in amoebae, we find greater discrepancies. Thus the effects of sodium and potassium on amoebocyte tissue can be shown to be markedly different if we compare various concentrations and osmotic pressures of the salts of these metals with chlorides, nitrates or even sulfates; potassium in sufficiently low concentrations appears to have a specific softening effect on the amoebocytes; it causes the taking up of fluid with an intensity not observed when sodium is used. In the case of the amoebae on the other hand no mention is made by various authors of such a difference. But in muscle tissue, Jacques Loeb recorded an effect of potassium which is in accord with our findings in amoebocytes. In the case of ammonium chloride a similar formation of mulberry-like forms was noted in amoebocytes by us and by Pantin in amoeba Limax. As far as calcium is concerned, Chambers, Pantin, as well as Spek, found a coagulating effect in the case of various amoebae, and Pantin and Chambers observed a similar, although less pronounced, action in the case of magnesium. Calcium therefore acts not unlike acid on these organisms. In the case of amoebocyte tissue, on the other hand, we found a broadening and flattening effect of calcium chloride which is soon followed by solution processes. Magnesium in its action on amoebocytes is intermediate between calcium and sodium. It is possible that also in amoebocytes these changes indicate that at certain stages processes of coagulation have taken place in the hyaloplasm of these cells.

THE FACTORS UNDERLYING AMOEBOID MOVEMENT IN BOTH AMOEBOCYTES AND AMOEBAE. ANALOGIES BETWEEN AMOEBOID MOVEMENT AND MUSCULAR CONTRACTION

If we consider how far the same factors underlie amoeboid movement in both amoebocytes and amoebae and wherein the differences observed in the two classes of organisms consist, we shall have to discuss as common factors

that may come into play (1) changes in consistency in the ectoplasmic layer as well as in the granuloplasm, (2) phenomena of contraction, and (3) surface tension changes.

(1) Changes in consistency in the protoplasm constitute in general the primary factors in amoeboid movement both in amoebocytes and amoebae. In amoebocytes a phase of softening is followed by a second phase of hardening in the ectoplasm, as well as in the intergranular hyaloplasm. In general amoeboid movement depends also in amoebae upon primary changes in consistency of certain constituents of the cell. However, in amoebae these changes are complicated through the existence of a distinct and preformed elastic membrane surrounding the cell. Associated with the presence of this cell structure are modifications in the way the rhythmic softening and hardening processes occur in various cases. In some amoebae they seem to depend on the ectoplasm-endoplasm transformation of Rhumbler. In other cases movements to a certain extent resembling those observed in amoebocytes seem to occur. In still other amoebae the rolling movement of Jennings constitutes the typical mode of locomotion. In this type of movement changes in consistency also occur in all probability, but to what extent they take place is uncertain. These differences between various kinds of amoebae depend probably upon primary differences in structure, in particular upon the relative rigidity and firmness of the membrane, and of the ectoplasmic and endoplasmic layers in these organisms. In some amoebae the consistency of the protoplasm can apparently be so low, at least under certain conditions, that the movement resembles that of a drop of fluid.

(2) We have seen that processes of contraction play a part in the amoeboid movement of amoebocytes under various conditions. It is even probable that they participate regularly in certain phases of amoeboid movement, causing a limited retraction of the pseudopod as well as of the granuloplasm and of the posterior end of the cell. Processes of a related character in amoebae have been described especially by Dellinger. According to our interpretation in amoebocytes, these phenomena depend upon a change in the character of the ectoplasmic and intergranular hyaloplasm of such a kind that during the process of gelation these protoplasmic structures not only gain elastic properties but are already in a state of elastic extension. Presumably this change is produced through the orientation of the micellae of the hyaloplasm in the direction in which subsequently the elastic retraction takes place. We have referred to the analogy which seems to exist between this process and the change which takes place in fibrinogen after its conversion into fibrin and we also referred to the apparent relationship between the contraction of certain parts in the amoebocytes and the retraction which takes place in the agglutinated amoebocytes and in the drawn out protoplasm of the latter in case the blood has been obtained through an incision in a joint and has been allowed to flow over the surface of the Limulus into the vessel. The retraction is more restricted, but it still occurs, due to an elastic pull which develops when amoebocyte tissue is prepared from Limulus blood obtained with such precautions that there is the least possible disturbance of the cell. Elastic stress develops under these conditions unequally in certain directions; the lines of stress are determined by the manner in which the amoebocytes or the protoplasmic substances of disintegrated amoebocytes became oriented to each other at the time when these various parts agglutinated into one mass. Under such conditions mechanical factors cause certain patterns to appear in the

blood fluid during the process of sedimentation of the amoebocytes. The latter are arranged in lines which follow a radial direction, all converging towards the center. Some of these radii are coarser than others, but they are all connected by concentric rings, also representing agglutinated amoebocytes. Thus a network is produced in the blood of Limulus and the thickest and strongest lines thus formed determine the direction of elastic retraction at a later period, when the cells have settled to the bottom of the vessel and have formed here an apparently homogeneous layer. The persistence of the physical characters of the protoplasm which are responsible for the development of the elastic strain depends upon the action of Limulus serum, which preserves for some time at least a certain required colloidal state of the hyaloplasm. If we replace the layer of serum by other solutions, like isotonic sodium chloride, neutral, acid or alkali in reaction, the colloidal condition of the hyaloplasm is changed to such an extent that the elastic strain no longer develops. The latter media make the hyaloplasm either too hard or too soft, and they thus interfere with its ability to gain elastic properties. This change in the physical character of the amoebocyte tissue under the influence of various media can in certain cases be directly demonstrated. Thus if we rub a piece of ordinary amoebocyte tissue between two glass slides, sausage-like structures develop which are relatively brittle and which through continued rubbing can readily be separated into small dense particles of agglutinated and rolled together amoebocytes; but, on the other hand, if we manipulate in the same manner a piece of amoebocyte tissue which has previously been under the influence of an alkaline solution of 0 5 N NaCl, the hyaloplasm has softened under the influence of the alkali and it can now readily be drawn out into fibers; it has lost its brittleness and at the same time has become so soft that it is no longer able to develop a noticeable elastic pull and to retract.

(3) Surface tension changes may play a part in amoeboid movement. Movements of fluid within the cell occur in certain phases of the cyclic changes constituting amoeboid activity. They are quite apparent during balloon formation and in circus movement. Similarly in cases in which fluid penetrates suddenly from the outside into a pseudopod and rounds it out so that it becomes a balloon, surface tension changes are at work. Likewise in certain types of amoebae there may be at times so great a fluidity of the protoplasm that the amoeboid movement seems essentially to depend on the movement of liquid in accordance with the laws of mechanics. But all these conditions are found either in cells undergoing abnormal amoeboid movement or they occur only in certain types of organisms at certain times. Whether in the ordinary amoeboid movement surface tension changes are of great importance is doubtful. In the ectoplasm as well as in the granuloplasm the smallest particles, constituting this colloidal material, seem to be in firmly oriented relation to the neighboring particles or at least their mobility in relation to each other is very limited and not of a magnitude characteristic of the movement of fluids. However, it is probable that in the first phase of pseudopod formation the softening process may in certain cases be sufficiently strong to allow surface tension changes to become a factor in this process; the same may apply also to the movement of the granuloplasm; here also there may occur processes of temporary softening sufficiently pronounced to allow surface tension changes to assert themselves. However, such changes would represent merely a temporary condition and one which is secondary to alterations in the consistency of the hyaloplasm.

We thus see that the amoeboid movement is a process of much greater complexity than was assumed by the earlier investigators. It was then believed that surface tension changes of a drop of fluid represented the essential features of amoeboid movement. While we have now to a certain extent a better understanding of the mechanisms underlying amoeboid movement, yet we must admit that the chain of facts established so far is not complete and that in many cases it must be supplemented by inferences in order to fill gaps in our knowledge concerning certain phases of the process, which do not readily lend themselves to analysis by observation and experiment.

While our understanding of the mechanism of amoeboid movement is more complete than that of the mechanical processes underlying muscular contraction and relaxation, it is not so satisfactory as far as the thermodynamic and chemical factors, which are at the base of the latter processes, are concerned; here the investigations especially of Hill and Meyerhof have gone far toward explaining these aspects of the problem. In the case of amoeboid movement on the other hand nothing definite is known in this respect except that amoeboid movement takes place in response to a stimulus. However, we may surmise that amoeboid movement is initiated and accompanied by certain metabolic changes localized in certain areas of the cell and propagated from here to adjoining places. We may furthermore surmise that in analogy to the part played by lactic acid in muscle contraction the stimulus causes, also in amoeboid movement, the formation of an acid, perhaps of lactic acid at the anterior pole and that it is the production of this acid which initiates the formation of a pseudopod.

In a somewhat different manner O. Fürth has attempted to coordinate the mechanism of muscular contraction and of amoeboid movement. He assumes in both these processes a combined action of surface tension and of imbibition of water on the part of the protein granules. The increased size of the granules, caused by the imbibition of fluid, in addition to the lowered surface tension induced by solution of the proteins, of which the granula consist, is according to this view responsible for the sending out of a pseudopod. However, such a mechanism it seems does not explain the localized changes in the colloidal state of the hyaloplasm and the rhythmic sequence in the movement of the ectoplasm and of the granuloplasm on which, as we have seen, amoeboid movement in amoebocytes primarily depends. Yet notwithstanding this difference also Fürth attributes to the action of acid on proteins an important rôle in the mechanism of amoeboid movement.

Amoeboid Movement in Tissue Cells of Higher Organisms

If we consider amoeboid movement in higher, multicellular organisms, it is probable that the character of the movement of leucocytes and of certain other wandering cells, which are found in the interstitial connective tissue, is similar to that described by us in amoebocytes. While so-called fixed cells in the fully developed organisms are normally without amoeboid activity, under special conditions a number of tissue components may acquire the ability to move. Such a change in the behavior of fixed cells takes place if stimuli of an unusual character reach certain tissues. This effect is, for instance, observed in the process of regeneration during wound healing, in so-called inflammatory conditions, or, after transformation of tissue cells into tumor cells. In these cases it is necessary that, previous to the assumption of amoeboid activity, the normal structural connections between neighboring cells which compose

the tissues be severed, so that these cells are at this stage joined together merely through agglutination. Under the further influence of certain stimuli these tissue cells may then separate through amoeboid movement and carry out active locomotion, such as we find it for instance in epidermal cells during regeneration or during their growth in culture media. These cells act thus similarly to the amoebocytes, which detach themselves from amoebocyte tissue. In a corresponding manner other tissue cells which normally are in an extended condition, as for instance, endothelial cells lining capillaries or sinusoids, may contract, separate from the surface on which they rest and move away.

While thus certain complicating factors exist in the case of tissue cells of higher organisms, they may under certain conditions essentially behave in a manner similar to amoebocytes which form a part of amoebocyte tissue.

Phagocytosis

By phagocytosis we understand the taking up on the part of living cells of formed, usually microscopically visible particles from the surrounding medium into the interior of the cell. Many cells have the faculty of phagocytosis; free-living amoebae as well as cells freely movable within a complex organism, such as polymorphonuclear leucocytes or the reticulo-endothelial cells; but even many of the so-called fixed cells can act as phagocytes, either in a stimulated state, as for instance, during the process of regeneration or when growing as tumor cells, and also in an apparently quiescent state, although they are less active than the normally motile cells. Cells which have a relatively large amount of cytoplasm, which are motile and have the proper consistency of their protoplasm, are especially prone to act as phagocytes. The kind of material which is preferably taken up also varies in the case of different types of cells.

Referring to the fact that especially motile cells tend to be phagocytic, there is observed a certain relationship between amoeboid movement and phagocytosis, but this does not imply that all amoeboid cells must therefore act as phagocytes; thus, for instance, the amoebocytes of Limulus do not readily phagocytose foreign particles which they encounter when wandering under the cover glass. Evidently some additional conditions have to be fulfilled before the cells become efficient as phagocytes. On the other hand, as we stated above, cells which are apparently non-movable and in a fixed state and which are constituents of a larger structural unit, such as the epithelial cells of the thyroid lining the lumen of an acinus, may take up foreign particles. Here the mechanism of phagocytosis must be somewhat different from that observed in amoeboid cells. Even epithelial cells, which in their normal fixed condition do not take up foreign particles, may do so actively when they begin to regenerate and carry out amoeboid movement. Thus we could show that epidermal cells may take up particles of coagulated blood serum; and it is probable that, in this case, phagocytosis depends upon active amoeboid movement. After the particles have been taken into the interior of the cell, they may undergo often further changes; if possible they are digested or they otherwise disintegrate; but in some instances if they prove unchangeable, they may later be discarded by the cell.

As to the process of phagocytosis, the conceptions which prevailed at various periods regarding the state of the protoplasm and the character of amoeboid movement largely influenced the theories concerning its mechanism.

The same authors who referred amoeboid movement to surface tension changes of drops of fluid likewise suggested that phagocytosis depends on similar processes at the surface of the fluid protoplasm. Thus Rhumbler believed to have reproduced the essential features of phagocytosis in drops of fluid, in which he induced localized changes in surface tension and cohesion, provided the chemical composition of particle and fluid were such that they could adhere to each other. Thus a drop of chloroform will draw into its interior a thin glass rod coated with shellac; chloroform can mix with shellac, dissolve it and therefore adhere to it. After the shellac has been dissolved, the glass rod is expelled from the drop, because the cohesion between the molecules of chloroform is greater than the adhesion between the chloroform and the glass rod. During the process of phagocytosis, the surface tension of the fluid is lowered at the place of contact between drop and foreign body and a spreading out of the fluid on the surface of the foreign body occurs. Such were the original conceptions of Rhumbler, although the observations of other authors and his own later observations induced him to admit subsequently certain complications, depending upon the presence of solid surface layers surrounding some cells. He therefore added to the action of surface tension and adhesion, preliminary solution processes in the surface film of the cell or the action of what he designated as gelatinization pressure, by which he evidently understood the pressure exerted by a solid membrane held under elastic tension by the fluid interior of the cell; but notwithstanding these complications, surface tension changes and adhesion to the foreign body remained now as before, for Rhumbler, the principal factors in phagocytosis. A number of later investigators likewise accounted for this process on the basis of the mechanics of liquid substances; the relative simplicity of such a process seemed very attractive and invited even a quantitative treatment of the observations made. Thus, Tait especially, and, with some reservations, also Fenn attempted an analysis of phagocytosis on this basis. However, the latter author recognized some discrepancies between the outcome of experiments and the requirements of a theory of phagocytosis, starting with the premises of a fluid character of the protoplasm, and in his later writings he therefore acknowledged changes in consistency of protoplasm as a factor in this process.

The principal facts established concerning the factors influencing the intensity of phagocytosis are compatible with the conclusion that changes in the colloidal state of the cell constituents are essentially responsible for this activity. Thus certain substances, opsonins, are produced through immunization against organisms, these opsonins acting by combining with the cells to be phagocytosed. They may perhaps increase the stickiness of the latter and thus facilitate their adhesion to the phagocytes and in general it is found that agglutination precedes phagocytosis.

The optimal temperature for phagocytosis is apparently not the normal body temperature of mammals at which other vital functions have their optimum, but it seems to be below this point; this fact suggests the conclusion that the viscosity either of the medium in which the cells are suspended or the viscosity of the phagocytosing cell must not reach below a certain level in order to obtain the greatest phagocytic activity.

As to the optimal hydrogen ion concentration, Fenn has shown that it is situated slightly on the acid side of the neutral point. Again it may perhaps be possible to interpret this fact as indicating that the viscosity of the proto-

plasm of the phagocyte, at the point of highest efficiency, should not fall below a certain level. Hamburger, Hekma and de Haan observed that addition of calcium salts increases phagocytic activity. However, recent experiments of Radsma and also of Fenn seem to indicate that calcium in this case merely has the function of replacing the calcium salts, which have previously been withdrawn from the medium or the cells through the use of oxalate or citrate or even of NaCl solutions.

In contrast to the requirements for amoeboid movement, the presence of colloids in the medium does not seem to be necessary for phagocytosis. Whenever the particle to be taken up agglutinates with the cell, the processes necessary for phagocytosis can evidently proceed; and this may take place even in a sodium chloride solution. According to Hamburger certain lipoid soluble substances, like chloroform or even small amounts of soap, may be favorable for phagocytosis. Hamburger assumed that their action depends on their surface tension lowering effect. However, it is not certain that this is the only possible interpretation of these observations.

In general, we may conclude that amoeboid movement and phagocytosis have a number of factors in common, and that in both processes changes in the colloidal state of cell constituents play a most important part; but that on the other hand there are certain other factors which are specific for amoeboid movement and for phagocytosis. In both conditions a localized softening on the surface layer of the cell in contact with a foreign body, seems to be the primary factor, leading in one case to the agglutination between phagocyte and the object which is to be phagocytosed, and ultimately to its inclusion in the cell body, and in the other to the sending out of a pseudopod. In certain cases such a localized lowering of the surface layer may perhaps suffice for the initiation of phagocytosis without the subsequent cooperation of amoeboid movements being required. As to the factors which determine the readiness with which certain amoeboid cells assume the character of phagocytes, it is probable that a certain cell consistency exists which is optimal for phagocytosis and that the range of variations in cell consistency under which phagocytosis occurs, is more limited than the corresponding range in the case of amoeboid movement.

EXPERIMENTAL TISSUE AND AGGLUTINATION THROMBUS

In some of our earlier papers on amoebocytes of Limulus we referred to the significance of some of the changes taking place in amoebocytes under abnormal conditions for the analysis of tissue formation: we referred especially to the transformation of agglutinated masses of amoebocytes into fibrillar structures under the influence of mechanical factors. Subsequently we attempted an analysis of some of the conditions underlying tissue formation by initiating processes in amoebocyte tissue leading to the formation of various tissue-like structures through the modification of environmental factors.

We have referred to the spreading out of amoebocytes in contact with solid surfaces and the subsequent contraction which may take place in certain cases in response to stimulation, processes corresponding to the flattening out of certain tissue cells as, for instance, endothelial cells, which also in the case of stimulation may contract and subsequently detach themselves from their base and migrate. In both amoebocytes and tissue cells of higher organisms, stereotropism is one of the factors that determines the direction of the movement. We observed that stereotropism depends upon the

necessity of contact with a solid base if amoeboid movement is to lead to effective locomotion. In the case of amoebocytes we were able to analyze the agglutination process associated with normal amoeboid movement and also the conditions of extension and subsequent contraction to which we referred above. We found it possible to initiate certain phases of wound healing in vitro and to observe in amoebocyte tissue the centrifugal outgrowth which corresponds to that seen in cultures of higher tissues. Our conclusion was that this mode of outgrowth in the case of amoebocytes is a chance phenomenon, resulting from the tendency of amoebocytes to free themselves from cells to which they adhere, and subsequently to maintain for some time unchanged the state of cell polarization and consequently the direction of their migration. Inasmuch as the chances to meet other amoebocytes become proportionally smaller the further the distance between the piece of tissue and the wandering cell, growth must take place in a centrifugal direction. In addition we have referred to the relation which exists between the secondary changes of a degenerative character in outgrowing amoebocyte tissue and tissue differentiation in general and to the similarity between certain formations observed in the former and in mesenchymatous or epithelial formations of higher tissues.

So far it has been possible to produce experimental amoebocyte tissue only with the blood of Limulus. Several factors make the blood of this animal particularly suitable for this purpose: (1) It can readily be obtained in large quantities and by the use of certain precautions injury to the amoebocytes during the process of collection can be restricted. (2) A real coagulation does not occur in Limulus blood and (3) it contains only one kind of cell. Recently Fauré-Fremiet has made use of agglomerations of blood cells of Arenicola in somewhat similar tissue culture experiments. As to the mechanism underlying the centrifugal growth, this author believes that currents which develop in a centrifugal direction at the air-fluid boundary and which can be demonstrated through the movements of particles of talcum powder suspended in the fluid, carry the cells along in a passive manner. The character of the centrifugal movement would thus differ in the case of blood cells of Limulus and of Arenicola.

Of particular interest in connection with the formation of experimental tissue is the behavior of isolated sponge cells and their tendency to unite with other cells in order to form tissue-like structures and complete new organisms because these observations supply confirmation for our conclusion that the phenomena of agglutination and subsequent changes which can be observed in amoebocytes of Limulus are basic factors underlying tissue formation in general. These agglomerations of sponge cells represent a kind of tissue slightly more complex, as far as the mechanism of its formation is concerned, than experimental amoebocyte tissue, but the principles relating to the production of the tissues are in both cases the same. Subsequently in the case of the sponges, some complications are added to these primary factors. H. V. Wilson first separated sponge cells from each other and was able to observe that these cells later coalesced again, thus forming aggregates from which complete organisms could develop. More recently Galtsoff, observing more closely the behavior of these isolated cells and their mode of aggregation, noted that it is the archaeocytes which play the principal rôle in this process and that they resemble in their behavior isolated amoebocytes. In both cases the migrating cells happen to meet other cells of their kind in the course of their

movements and whenever this takes place they stick to them. We may therefore consider agglutination as the primary process underlying tissue formation, and it can be shown that this process of agglutination is not due to the deposition of a protein substance upon the periphery of the cell, but to a change in the consistency of the hyaline ectoplasm of the amoebocytes or of corresponding cells. Thus we have to deal with a process of agglutination comparable, for instance, to the sticking together which occurs in certain bacteria under the influence of acid, a process which in both cases is commonly designated as agglutination. The similarity between the behavior of sponge cells and of amoebocytes goes still further, inasmuch as, according to Galtsoff, there is in the movement of archaeocytes the same lack of an orienting force determining the direction in which these cells will move, which we have noticed in the case of the amoebocytes; in both types of cells we have to deal with chance movements of amoeboid cells. In both cases, the primary agglutination and the stickiness of the hyaline ectoplasm underlying this latter process, are related to the factors concerned in the production of pseudopods and in the extension of the cells. Temperature, osmotic pressure and hydrogen ion concentration apparently affect amoebocytes and archaeocytes in a similar manner; for instance, alkali seems to increase the tendency of both kinds of cells to agglutinate. However, as far as the action of salts and the ions composing them are concerned, the reactions of the archaeocytes seem to correspond more closely to those observed in the case of free-living protozoa than in the case of amoebocytes which latter are adapted to a protein containing environment.

We may therefore regard the experimental amoebocyte tissue as representing the most primitive and rudimentary type of a tissue and the sponges as the next higher grade in which a further differentiation of the component cells and their power to proliferate is superimposed on this primitive mode of tissue formation.

The agglutination process, which underlies this tissue formation, occurs in cells which under usual conditions circulate within the blood channels of the animal. Normally, they do not agglutinate; but if a foreign body is introduced into the blood channels the same factors, which lead outside the body under definite conditions to the formation of the prototype of a tissue, lead inside the vessels to the formation of an agglutination thrombus. The same change in consistency of the cells, called forth by similar environmental alterations, underlies both these processes. From here on through the whole series of higher organisms we can follow the agglutination of certain elements of the blood in vitro, preceding coagulation of the blood, and in vivo, leading to thrombus formation. The spindle cells of the lower vertebrates and the blood platelets of mammals correspond to the amoebocytes but the essential process underlying these changes is the same in all of these classes of animals. Changes in the environment of the cells caused by injury of the vessel wall, as for instance, by the introduction of a foreign body exerting mechanical effects on the blood cells, associated with a slowing of the circulation, lead to the formation of agglutination thrombi. Thus the basic factors leading to the formation of the most primitive tissues and of agglutination thrombi are the same and both primitive tissues and agglutination thrombi are the same and both primitive tissues and agglutination thrombi find their prototype in the amoebocyte tissue and these two processes gradually undergo various developments and complications in the more complex classes of organisms.

The Physical Basis of Life *

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Huxley's famous address on the "Physical Basis of Life," delivered at Edinburgh in 1868, was originally published as one of his *Lay Sermons, Addresses and Reviews*. In this discourse, a classical model of popular scientific exposition, the eminent English biologist first brought to the attention of lay readers the significance of protoplasm considered as the physical substratum of vital action. It was in substance a popular presentation of conclusions that had gradually been taking shape as the result of experimental and microscopical researches by such investigators as Max Schultze, De Bary, Cohn, Brücke, Kühne and Lionel Beale. Those conclusions, originally published in technical form, were at that time almost unknown to the general public. Huxley's exposition of them at once caught the popular ear, partly because of its lucid and captivating style, partly because of the rough jolt that it gave to popular conceptions concerning the nature of life. It aroused a storm of criticism and protest which was intensified a few years later when Tyndall, in his famous Belfast address, proclaimed his faith in non-living matter as offering the "promise and potency of every form of terrestrial life." The storm was little more than a sign of growing pains and in due course subsided. Huxley's heresy of sixty years ago gradually became respectable and orthodox doctrine; but the problems of protoplasm still hold us fast with a gripping interest that has lost nothing of its force with the flight of time. In what light do Huxley's conclusions appear after the biological progress of half a century?

Modern biologists long since accepted those conclusions, not in any spirit of dogmatism or finality, but because in practice they provided a highly useful programme for precise investigations on the phenomena of life. In one important respect, however, an exception must be taken to the form of Huxley's presentation. His words seem clearly to imply, if they do not actually state, that protoplasm is a single chemical substance or "living protein". In his opening words he speaks of the physical basis of life as "some one kind of matter common to all living beings". He pictures a union of lifeless substances, such as water, ammonia and carbon dioxide, to form "the still more complex body, protoplasm"; and the properties of this substance, he affirmed, must result from the nature and disposition of its molecules. "The thoughts to which I am now giving utterance," said Huxley, "and your thoughts regarding them, are the expression of molecular changes in that matter of life which is the source of our other vital phenomena."

It is necessary to bear in mind that these words were written sixty years ago, before the complex organization of the cell had been revealed. We can

no longer state the matter so simply. What we now mean by the word "protoplasm" is certainly not any single or homogeneous chemical substance. The term designates a heterogeneous and highly complex mixture of many diverse substances and one that is the seat of incessant chemical transformations. The cell is an intricate living system, largely composed of matter in the colloidal state and hence properly designated as a *colloidal system*. With the microscope, aided by other technical methods, we distinguish in this system a continuous, clear, viscid and seemingly homogeneous ground-substance or *hyoplasm*, in which are dispersed a great variety of *formed bodies*, varying widely in shape, number and physico-chemical nature, and often grouped in characteristic patterns. Each of them plays no doubt some definite part in the cell-activities, though on the whole we are still surprisingly ignorant on this subject. Examples of these bodies are the nucleus (itself a complicated system), the plastids, especially characteristic of plant-cells, where they serve as centers of specific chemical activities; the chondriosomes, apparently of universal occurrence, but still of doubtful function; the Golgi-bodies, now believed to play a leading rôle in secretion and perhaps in the production of organic enzymes generally; vacuoles; and in addition many kinds of granules and fibrillae, differing widely in form, size, physical and chemical nature, and in function. Some of these bodies seem to be permanent and self-perpetuating, others to be transitory formations that come and go in the kaleidoscopic operations of cell-life. Which of the components in this diversified assemblage are alive? Which represent the physical basis of life? What, exactly, is protoplasm?

These are natural but somewhat misleading questions. The truth is that the more critically we study the question the more difficult does it become to single out any one particular component of the cell as the living stuff *par excellence*. Of this fact most experienced cytologists, including such eminent leaders as Fleming, Strasburger, Bütschli, Kölliker and Heidenhain, long since became convinced. "No man," said Fleming, "can definitely say what protoplasm is. . . . In my view that which lives is the entire body of the cell." As our knowledge of the cell has advanced, this conception of the physical basis of life has come more and more to the front, in the minds alike of physiologists, chemists and cytologists. I quote a distinguished biochemist. "We can not." says Professor Hopkins, "without gross misuse of terms, speak of the cell life as being associated with any particular type of molecule. Its life is the expression of a particular dynamic equilibrium which obtains in a polyphasic system. Certain of the phases may be separated, but life is a property of the cell as a whole, because it depends upon the equilibrium displayed by the totality of coexisting phases." In the words of Czapek, what we call life is a complex of innumerable chemical reactions in the substance of the system. I repeat, therefore, that when we speak of protoplasm as the physical basis of life, we mean simply the sum total of all the substances that play any active part in the cell processes; and we can not, I think, exclude from the list such substances as water and inorganic salts which we commonly think of as "lifeless".

Protoplasmic systems appear to us in two aspects, both involving the same fundamental problem, but in present practice approached by somewhat different methods. In one aspect the cell appears as an existing group of phenomena that may be directly examined by the experimental methods of the physicist and the chemist. It is here especially that modern biophysics, bio-

chemistry and the so-called colloid chemistry are beginning to play so important a part in the study of protoplasm, directly by experiment, and indirectly by studies on non-living heterogeneous systems, such as emulsions and colloidal solutions, which to a certain extent are made to serve as simplified models of living systems. Modern biology has already profited so much by investigations of this type—for example by studies on the properties of surface films and interfaces, on permeability, changes of viscosity and the like—as to suggest that investigations on protoplasm and cells may be destined to pass hereafter more and more into the hands of the physiologist, the physicist and the chemist. Certainly the steadily rising tide of research in this direction is of good augury for future progress in the experimental analysis of vital phenomena.

In a second and equally important aspect the cell appears, not merely as an existing colloidal system, but one that is *self-perpetuating;* for every cell arises by the division of a pre-existing cell, and by this process the entire heredity of every species of plant or animal may be transmitted. In theory, of course, this second or genetic aspect of cell-systems cannot be separated from the first—the two are indeed no more than different sides of the same fundamental problem. In practice, however, phenomena of this type have as yet been comparatively little explored by direct, exact and quantitative physico-chemical methods. . The main approach to them has been opened especially by the cytologist, the embryologist, and the geneticist; and it is to the problems here encountered that we ask attention.

The cytologist is first of all struck by the extraordinary pains that nature seems to take to ensure the perpetuation and accurate distribution of the components of the system in cell-division, and hence in heredity. An impressive demonstration of this is offered by the nucleus of the cell. To our limited intelligence, it would seem a simple task to cut a nucleus into equal parts. The cell, manifestly, entertains a very different opinion. Nothing could be more unlike our expectation than the astonishing sight that is step by step unfolded to our view by the actual performance. The nucleus is cut in two "meristically", that is to say, in such a manner that every portion of its net-like inner structure is divided with exact equality between the two daughter nuclei. The cell performs this spectacular feat with an air of complete and intelligent assurance. The nuclear substance is spun out into long spireme-threads or *chromosomes;* these are split lengthwise into exactly similar halves which shorten, thicken, separate and pass to opposite poles; and from the two groups thus formed are built up two daughter-nuclei, while the cell-body divides between them. In outward appearance such a process seems to contradict all physical principles, but its meaning has now become perfectly plain. In a general way it means, as Roux pointed out on theoretical grounds forty years ago, that the nucleus is not composed of a single homogeneous substance, but is made up of different "qualities" or components, which are strung out in linear alignment in the threads to be divided and distributed in a particular manner through the longitudinal doubling of the threads.

Roux's fundamental conception has been confirmed by a series of investigations that have brought forth some of the most notable discoveries of our time. The direct cytological evidence of a serial alignment of smaller bodies along the nuclear threads thus far indicates the fact only in a somewhat rough fashion, but clearly shows that the nuclear threads often contain smaller bodies or "chromomeres" aligned in a single series, and sometimes showing an ap-

proach to constant size-differences and a definite serial order. This visible structure, as viewed in the coagulated material of thin sections, is however no more than the rough expression of a finer one that lies beyond the reach of the microscope. This conclusion is made highly probable, if not directly demonstrated, by researches on a grand scale on the mechanism of Mendelian heredity carried out especially by Morgan, Sturtevant, Bridges and their co-workers in their widely known experimental studies on heredity in the fruit fly *Drosophila.* This animal offers unparalleled opportunities for researches of this type. It can easily be bred under standardized conditions. It produces numerous offspring. Its development takes place with remarkable speed. It frequently produces heritable mutations. The researches thus made possible have demonstrated the fact, established in a more general way by earlier observers, that the nuclear threads of chromosomes play an essential part in heredity; and they have removed every doubt that the Mendelian phenomena may be fully explained by the behavior of these bodies or their components (as was first indicated in a more general way by Sutton, Boveri and De Vries).

This, however, was only the beginning. Beyond all this, these investigations have brought overwhelming confirmation of the correctness of Roux's conception of the nuclear threads as linear aggregates of smaller entities. The precise nature of these entities is still unknown; hence they are provisionally called "gens"; in any case they are of extreme minuteness and may possibly be single molecules of protein. The genetic evidence leads rigorously to the following series of conclusions concerning the gens: They differ specifically among themselves and are self-perpetuating, each after its own kind; they may be combined and recombined in many ways without loss of their identity; each stands in some kind of special causative or determinative relation to a corresponding particular, hereditary character, though the action of each gen may be modified by that of others, and many or perhaps all the gens may thus coöperate in determination; they are aligned in the spireme-threads in a definite serial order; and they may conjugate and disjoin two-by-two in perfectly orderly fashion, thus providing the physical mechanism that lies behind Mendel's laws of heredity.

The first reaction to this series of conclusions—so remarkable and seemingly artificial—was a feeling of skepticism; nevertheless they have quickly won their way to general acceptance on the part of genetical experimenters. It is well to emphasize the fact that these conclusions did not arise as *a priori* constructions. They were not born in the fertile imagination of a Bonnet, a Buffon or a Weismann. They were products of concrete and far-reaching experiments under carefully controlled conditions; they make possible precise and quantitative prediction; and the data on which they rest can be confirmed by laboratory experiment almost as readily as those on which the physicist or the chemist bases his conceptions of the molecular and atomic structure of matter. In these respects they are comparable in validity with many of the fundamental concepts of modern physical science. In theory, no doubt, it is possible to consider them as merely a convenient fiction or algebraic symbolism, a kind of ideal model by means of which the genetic facts may conveniently be grouped and analyzed. But genetic phenomena are not mere abstractions or formulae. They are concrete expressions of the activities of cell systems; and it is more in accordance with modern scientific methods to make use of the actual model which every dividing cell displays to us in visible reality—a model that is not less impressive because at present the

microscope of the cytologist can only make visible its broader outlines with no more than dim indications of the finer complications inferred from the results of genetic research. And in point of fact all leading investigators in modern genetics have accepted this actual model because of its immense productiveness in discovery. In this respect the theory of the gens as applied to the nucleus deserves to stand beside the atomic theory as employed in modern chemistry and physics.

Cytology and genetics have thus combined to demonstrate an organization in the nuclear region of the cell-system that is as complex and wonderful as any pictured by the fantasy of the speculative nature-philosophers. But we cannot stop here. Willy-nilly we are led on to the question whether an organization of similar type, or in any degree approaching to it, may also exist in the cell-body or cytosome. Conservative opinion, both cytological and genetic, has been extremely reluctant to recognize such a possibility. We have been apt to take the cytoplasmic region of the cell-system at its face value; to think of it as devoid of definite organization, or organized only by the domination of the nucleus. We have been accustomed to think of division of the cytosome as a simple mass division contrasting fundamentally to the complicated meristic process of nuclear division. Observations are however slowly accumulating which suggest a reconsideration, and possibly important modifications, of this notion; for it has been shown that many of the cytoplasmic components are directly transmitted from mother-cell to daughter-cell in the shape of pre-existing formed bodies.

Vague indications of such a conclusion were long since given by the plastids of plant-cells, which are certainly in many cases, perhaps in all cases, self-perpetuating by growth and division without loss of their identity. Later it was demonstrated that the same is true of the centrioles or central bodies, which form the foci of mitotic cell-division. More recent cytological inquiry raises the question whether the principle of genetic continuity may not be of wider applicability in the cytoplasmic system. It is known that the chondriosomes, the Golgi bodies and the vacuoles (according to some observers identical with the Golgi system) have remarkable powers of growth and of multiplication by some kind of division or fragmentation. It has been shown that in a large class of cases they, or the materials of which they consist, are handed on during division from mother-cell to daughter-cell. In many cases, it is true, this process shows no evidence of an exactness of distribution comparable with that seen in nuclear division; it gives rather the impression of a hit-or-miss process, a merely incidental or mechanical result of the division of the cytosome. There are furthermore a few cases of this type (*e.g.,* in certain scorpions) in which the distribution is demonstrably inexact. Such cases nevertheless are connected by intermediate conditions with others in which the chondriosomes and Golgi bodies enter into a more definite association with the mitotic figure. In the latter cases the distribution becomes more accurate; and in one well-determined case (spermatocytes of the scorpion *Centrurus*) all the chondriosomes become aggregated into a single ring-shaped body that is divided and distributed in the course of the ensuing division with such exactness that when it was first discovered it was actually mistaken for a chromosome.

Such facts prominently raise the question whether the chondriosomes and Golgi bodies, and possibly various other components of the cytoplasmic system, may not be self-perpetuating, or composed of self-perpetuating material. The

possibilities of genetic continuity here observed have not yet been sufficiently explored to warrant extended discussion at present; but in the writer's opinion they should be kept in view in every consideration of protoplasmic systems. Here, however, an important distinction must be drawn. The self-perpetuating central bodies and plastids evidently are individualized, and perhaps organized, structures, but it is more than doubtful whether the same can be said of the chrondriosomes and Golgi bodies. The facts seem indeed clearly to indicate that these bodies are not sharply individualized but are rather, in each case, variable and plastic forms of a specific material, capable of extensive and possibly unlimited growth, and often handed on unchanged from cell to cell during cell-division. The interesting possibility is here suggested that it is this *material,* rather than the individual bodies which it forms, that may be self-perpetuating; and the broader possibility is also indicated that the same may be true of other components of the cell-system. Let us look into these possibilities a little further.

It is a widely prevalent view that many of the formed bodies arise *de novo,* being built up anew in the hyaloplasm by localized processes of chemical and morphological synthesis. Some recent observers have produced evidence that this may be true of the chondriosomes and Golgi-bodies; and it has even been held that under certain conditions plastids and central bodies may likewise have such an origin. Some of this evidence seems fairly convincing, some inadequate; but in either case it leads us to consider the implications of the phrase "formation *de novo.*" I will illustrate this by reference to some old observations of mine on those classical objects for the study of protoplasm the transparent eggs of certain sea-urchins and star-fishes.

As was long ago described by Bütschli, the protoplasm of these eggs when mature shows with great beauty the so-called "foam-structure" or "alveolar structure," *i.e.,* one which offers an emulsion-like aspect owing to the presence of innumerable dispersed spheroidal drops or bodies suspended in a clear, continuous semi-liquid basis or hyaloplasm. These bodies are of two general orders of magnitude, namely, larger spheres or *macrosomes* rather closely crowded and fairly uniform in size, and smaller *microsomes* irregularly scattered between the macrosomes; and among these are still smaller granules that graduate in size down to the limit of vision with any power we may employ. At first sight these various bodies seem to differ only in size; but this is illusory. It has been conclusively shown by various methods that in these eggs, as in other cells, the dispersed bodies are certainly of several kinds, including chondriosomes, Golgi-bodies, fatty bodies, yolk-spheres, sometimes pigment-granules, and perhaps many others. By Bütschli this "alveolar structure" was supposed to be an original and general characteristic of protoplasm; but this can no longer be maintained, as is proved by study of the growing young eggs. The smallest and youngest of them are composed largely of structureless hyaloplasm in which an alveolar structure first appears much later by the formation, growth and crowding together of numerous dispersed bodies. A small number of these, including Golgi-bodies and chondriosomes, are present from a very early stage and perhaps may be received from the mother-cell, subsequently multiplying, growing, enlarging, spreading through the cytosome, and becoming more crowded. Others, on the other hand, apparently including the macrosomes or yolk-spheres, arise by the growth of smaller bodies which themselves seem to arise *de novo.*

The protoplasm of the growing egg during this process gives an interesting

picture. When viewed under a relatively low magnification (300-500 diameters) only the larger bodies are seen; but as step by step we increase the magnification, step by step we see smaller and smaller bodies coming into view, at every stage graduating down to the limit of vision. This remains true even with the highest available powers. One might compare the microscopical picture offered by such protoplasm to the telescopic picture of the sky. As more and more powerful lenses are used new and fainter stars continually come into view. The astronomer knows that each enlargement will bring into view stars previously unseen. The cytologist is equally sure that if the present limits of direct microscopical vision could be extended we should see disperse bodies still more minute; and the invention of the ultramicroscope has in fact made us directly aware of the existence in the hyaloplasm of numerous suspended protoplasmic particles too small to be seen directly by the ordinary microscope, but made evident by their diffraction halos when viewed by the so-called ultra-microscope in powerful reflected light. Clearly, therefore, the hyaloplasm is not a simple homogeneous substance but itself contains dispersed formed bodies beyond the reach of the ordinary microscope, somewhat as is the case with a simple colloidal solution. The cytologist finds it difficult to resist the conclusion that the invisible dispersed particles may be as highly diversified chemically as the visible ones, and of all orders of magnitude down to single molecules or beyond. When therefore we speak of formed bodies appearing *de novo* we refer merely to the visible portion of the series and are quite ignorant of their real beginnings. But manifestly it is illogical to affirm an origin *de novo* of any formed body because it first becomes visible at a particular enlargement, even the greatest at our present command. Here, clearly, is an enormous gap in our knowledge. All points to the conclusion that below the horizon of our present high-power microscopes there exists a region of invisible organization that may be even more complex than the visible one with which the cytologist is directly acquainted. We have as yet little inkling of what takes place in this region, least of all perhaps in respect to the localization of those synthetic physico-chemical processes that are involved in the so-called formation *de novo*.

We have now arrived at a borderland where the cytologist, the biophysicist and the colloid chemist are almost within hailing distance of each other—a region in which the coöperation between them may be destined to play one of its greatest future rôles. In the meantime one of the cytologists will perhaps do no harm by the surmise that "formation *de novo*" may sometimes mean no more than the emergence above the horizon of visibility of formed bodies previously too minute to be seen. It seems entirely possible that some of these ultra-microscopical particles may, like the nuclear gens, be self-perpetuating centers of growth and division; but here we are perhaps too far over the line that separates fact from fiction. In beating a retreat let it be remarked that I am not here suggesting a resuscitation of the old conception of the cell as an assemblage or colony of elementary organisms or primary vital units—we have at present little definite evidence for or against such a speculation—nor am I able to see how the possibilities here suggested are in any manner out of harmony with the conception of the cell as a colloidal system.

In turning to the embryological aspect of the subject we enter upon the final stage of our inquiry. Experiment seems clearly to have demonstrated

that the differentiation of cells is not directly determined by their nuclei but by their cytoplasm—*i.e.,* that the specificity of cells depends upon the nature of the cytoplasmic materials alotted to them by the process of cleavage. Displacement of the larger visible formed bodies by centrifuging eggs has relatively small effect on development, which indicates that cell-specificity is not determined by them but more probably by the hyaloplasm; and exact studies on the germ-forming regions of the eggs and their relation to the cleavage-process drive us to the conclusion that the hytaloplasmic materials are in many cases segregated and distributed to the cells according to an ordered system. How is the order of this system determined? What makes the developing egg-cell a unit? It is at this point that we encounter some of the most difficult of the problems offered us by the physical basis of life.

We try to disguise our present inability to answer these questions by taking refuge in learned phrases. We are forever conjuring with the word "organization" as a name for an integrating and unifying principle in the vital processes; but which one of us is really able to translate this word into intelligible language? We say pedantically—but no doubt correctly—that the orderly operation of the cell results from a dynamic equilibrium in a poly-phasic colloidal system. In our mechanistic treatment of the problem we commonly assume this operation to be somehow traceable to an original pattern or configuration of material particles in the system, as is the case with a machine. Most certainly conceptions of this type have given us an indispensable working method—it is the method which almost alone is responsible for the progress of modern biology—but the plain fact remains that there are still some of the most striking phenomena of life of which it has thus far failed to give us more than the most rudimentary understanding.

The nebulous state in which the whole concept of organization still remains is brought home to us when we attempt to deal with the fact that every organism either is, or at some time has been, a single cell. When it has come to full development the organism consists of coordinated parts, displaying a multitude of cunning devices—anatomical, physiological or chemical—that make provision for the harmonious cooperation of its activities and for its protection and maintenance. To this extent its organization is obvious and intelligible; and to the same extent the organism is clearly a piece of mechanism, a living machine. But let us review the building of this mechanism by following it backwards, step by step, to its starting-point. Step by step we find the intricate machinery of life vanishing before our eyes until nothing remains but a single cell, the egg. In the egg-cell, complex though it may be in its own way, not a trace seems to remain of the coördinating and unifying devices of the adult; but who will maintain that the egg is not as specifically organized and as truly alive as the adult to which it gives rise?

It is an old notion, to which modern research has given a certain deceptive semblance of plausibility, that the embryo is already present in the egg, blocked out, as it were, "in the rough" in the cytoplasm, so that development has only to impress upon it the finishing touches. Did this notion correspond with the facts, it might provide us with a short cut out of our difficulties; but the evidence now seems conclusive that the rough model, with the more than doubtful exception of one or two of its most general features, is itself the product of antecedent localizing operations of development. Certain main features of the localizing process are often perfectly evident before the egg

begins its cleavage into cells, and may in some cases be followed readily by the eye. It is an impressive spectacle that is offered by the egg when engaged at its work of blocking out the embryo, without visible tools or model, but with an uncanny air of deliberate purpose and mastery of technique that any human artist might envy.

What then constitutes the organization of the egg? No one has yet found an adequate reply. The embryologist, the cytologist, the physiologist and the biochemist, all of these alike have thus far only skirted the outermost rim of the problem. We cannot predict how far the cytologist of the future may be able to penetrate into it; but it would seem that sooner or later his way will finally be blocked by inherent limitations of the microscope determined by the wave-length of light. If we are ever to find our way into the inntermost arcanum of the cell, other methods must be employed; and we must marshal and coördinate all the resources of experimental embryology, genetics, biophysics and biochemistry. Many important discoveries in these various fields have in recent years advanced our knowledge of development; but they leave its central problem still unsolved. From this very source indeed came the facts on which Driesch, a distinguished pioneer in experimental embryology, based his famous argument against the mechanistic theory of development and in support of a new philosophy of vitalism. The rock on which the whole mechanistic conception of organization and development splits, he insisted, is the fact that a fragment of an egg may undergo complete development and produce a perfect dwarf embryo. In spite of Driesch's alluring presentation, his argument fails to convince us; nevertheless, it still awaits decisive refutation. All, indeed, now points to the essential correctness of his contention that at the real beginning of development, the cytoplasm of the egg is devoid of any discoverable structural pattern or machine-like configuration that foreshadows the plan of the future embryo. Not alone the structural details of the embryo, but the very plan on which it is built seems to be constructed anew in the course of development.

In turning once more to the cell-nucleus we meet with a problem of similar type. As has earlier been indicated, the development of particular characters somehow depends upon the presence in the nucleus of corresponding particular and separable units, or "gens". We know from the celebrated experiments of Boveri and his successors that normal development depends on the normal combination of these units. Genetic evidence is now rapidly broadening our views in this direction by the accumulating demonstration that no one of the gens plays an exclusive rôle in the determination of any single character. It has been made clear that the individual gen may affect the production not merely of one character but of many. Conversely the probability is shaping itself that the production of any single character requires the coöperation of several or many gens, possibly of all. Geneticists even seem ready to accept the statement that every gen may affect the whole organism, and that all the gens may affect each character. We begin to see more clearly that the whole germinal complex, the whole cell-system, may be involved in the production of every character. How then are hereditary traits woven together in a typical order of space and time? It is the same old puzzle, made more precise but not yet, so far as I can see, brought appreciably nearer to its solution. We are ready with the time-honored replies: It is accomplished by the "organism as a whole"; it is a "property of the system as such"; it is the result of "organization". These words, like those of Goldsmith's country

parson, are "of learned length and thundering sound." Once more, in the plain speech of daily life, their meaning is: *we do not know.*

Perhaps indeed a day may come (and here I use the words of Professor Troland) when we may be able "to show how in accordance with recognized principles of physics a complex of specific, autocatalytic, colloidal particles in the germ-cell can engineer the construction of a vertebrate organism"; but assuredly that day is not yet within sight of our most powerful telescopes. Does this mean that our reliance on mechanistic methods and conceptions is weakening? Is the door here open for a vitalistic philosophy which would refer the operation of the living system to an occult power—a *vis essentialis,* a directive force, a vital impulse, an archaeus or an entelechy. In imaginative moments many a biologist has felt the lure of such a philosophy; but he well knows that to yield to it would be to abandon the scientific method and to have done with the central problem once for all. For the scientific method is the mechanistic method. It is by its use that our whole knowledge of living things has step by step been built up in the past, and is being further enlarged today by the study of colloidal systems. No one can set a limit to future progress along this road. To say *ignoramus* does not mean that we must also say *ignorabimus;* and to maintain that observation and experiment will not bring nearer to a solution of the great riddle would be to lapse into the dark ages. Perhaps Professor Henderson is right when he expresses his belief that organization has finally become a category that stands beside those of matter and energy. Perhaps there is no problem or none that we can formulate without talking nonsense. Perhaps we should go no further than to record and analyze the existing order of phenomena in living systems, without losing sleep over the imaginary problem of a unifying principle. Let us politely salute these uncomfortable possibilities as we pass, without swerving from our way.

The Filtrable Viruses

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The term of filtrable viruses was originally used to designate a group of disease-producing agents which seemed to differ from all forms of living matters then known, in their minute size, by virtue of which they were able to pass through filters that were generally regarded as bacteria proof. But the discovery that not all bacteria are held back by filters of this order has rendered a definition of the filtrable viruses based on size alone inapplicable. It has been shown, as a matter of fact, that bacteria exist which are so small that this alone enables them to pass, and in the case of various spiral organisms it seems quite clear that the element of motility when coupled with a certain degree of flexibility and a transverse diameter slightly less than the effective pore size, may enable an organism to pass, irrespective of its length.

Aside from size, there is another factor, however, which distinguishes the filtrable viruses proper from other disease producing organisms, namely their unknown toxonomic position, for they are clearly not bacteria, nor are they protozoa in the generally accepted sense of these terms. As a group they are not cultivable and the majority are invisible with the instruments which are now at our disposal.

Under the circumstances it would seem appropriate to use the term filter passers to denote all those forms of living matter which at some stage, or at any stage of their life cycle are capable of passing the pores of the common diatomaceous and porcelain filters, be they pathogens or not, and to restrict the term *filtrable viruses* to those pathogenic filter passers which are neither bona fide bacteria nor protozoa. It is with the latter that we shall deal in the present paper.

Following the discovery of specific disease-producing bacteria in the early days of bacteriology, hope ran high that ere long all infectious diseases would be traced to the activity of organisms of this order. This expectation has not been realized. It was found that some infectious diseases were caused by protozoa, but that a considerable number remained, in the production of which neither group of organisms seemed concerned.

The idea that organisms of such minute size might exist, as to be invisible even with the most powerful microscopes, and that such might be responsible for some of the infectious diseases of unknown origin, was hence a very natural one. It was directly expressed already by Pasteur, when he was unable to demonstrate microorganisms of any kind in the course of his studies on rabies.

The discovery that disease-producing agents actually exist which are so small that they cannot be seen with the most powerful instruments, was

made by Iwanowski in 1899 in the course of his studies of the so-called mosaic disease of tobacco. He found that it was possible to infect healthy plants with the juice of diseased plants after filtration through dense porcelain filters, and Beijerinck who made the same discovery independently in 1897, further showed that the disease could be transmitted in series by starting with such filtrates, and accordingly concluded that the infective principle must be animate. In the same year also came the announcement by Loeffler and Frosch that the dreaded hoof and mouth disease of cattle was due to an agent of this type.

While the observations of Iwanowski in a plant disease had apparently attracted little or no attention, the discovery of an invisible or ultramicroscopic virus as the causative agent of an animal disease naturally created a great deal of interest, and the search for filterable viruses was henceforth carried on most actively, whenever or wherever a suitable opportunity offered. At the present time approximately fifty diseases are known to be caused by infective agents of this type, and in as much as some affect invertebrates as well as vertebrates, and plants as well as animals, it is not unlikely that future investigations will show still other diseases to be due to representatives of this new group of disease-producing agents.

It is interesting to note that even the lowest group of living organisms, so far as we know them, viz., bacteria, may suffer from diseases which are caused by agents of this order, for whatever future research may show to be the true nature of Twort's lytic agent and d'Hérelle's bacteriophage, so much is certain that the bodies in question are filter passers, which do not belong to any one of the usually recognized groups of living matter, and that they can bring about the destruction of susceptible organisms in series, in other words that they possess the essential attributes which characterize the filtrable viruses.

To what extent filtrable viruses may cause disease among the higher plants, remains to be seen. At the present time we know that the mosaic disease of tobacco, of the tomato, of the Jamestown weed, of cucurbits is due to pathogenic agents of this order, but there is as yet not sufficient evidence to warrant the conclusion that the occurrence of mosaic diseases in general implies the activity of agents of this type. The future study of the etiology of this large group of plant diseases, however, will prove of extreme interest, in as much as the elucidation of the nature of the causative agent or agents of some of these forms may throw light upon the nature of some of the filterable viruses proper, and more particularly upon the question whether some of them may not be inanimate.

While very little as yet is known of infectious diseases of invertebrates, filtrable viruses have been shown to be responsible for three economically very important diseases of insects, viz., the jaundice of silkworms, the wilt disease of the gipsy moth caterpillar and the sacbrood of bees.

Amongst the low class of vertebrates, viz., fishes, amphibia and reptiles, no diseases have as yet been traced to filtrable viruses, but amongst the disease producing agents affecting birds the filtrable viruses occupy a prominent position. Chicken plague, epithelioma contagiosum, certain malignant tumors affecting chickens, leukemia of chickens, and a curious epidemic disease occurring amongst blackbirds have been traced to such agents.

Of mammalian diseases a large number besides hoof and mouth disease are now known to be caused by filtrable viruses, such as the pleuro-pneu-

monia of cattle, cow pox, sheep pox, goat pox, the Pferdesterbe (horse sickness) of South Africa, Rinderpest, rabies, hog cholera, the swamp fever of horses, the catarrhal fever, heart water and Nairobi disease of sheep, Agalaktia contagiosa of goats, the benign type of papulous stomatitis of cattle and still others that are economically less important.

Amongst the pathological conditions affecting man, finally, the following have been traced to filtrable viruses: molluscum contagiosum, dengue, warts, pappataci fever, smallpox, trachoma, mumps, rabies, infantile paralysis, measles, inclusion blenorrhoea, the various types of herpes, and possibly epidemic encephalitis, chicken pox and common colds.

I have already pointed out that the filtrable viruses proper are neither bacteria nor protozoa in the ordinarily accepted sense of these terms, and the question naturally arises are they living matter at all.* This question in turn raises another question, viz., do bacteria and protozoa such as we know them, represent the lowest types of living matter. Leaving the filtrable viruses out of sight for the moment, we can only say that we have no evidence that lower forms exist, but we must admit that it is scarcely conceivable, that the unquestionably wide gap between animate and inanimate matter should be barren of still lower forms than those with which biologists have thus far been familiar. A priori one would expect such *ultra-protists,* as they might properly be termed, to occur widely distributed in nature, but attempts to demonstrate them either by ultramicroscopic examination, by culture or by their effect upon animals of various kinds, as well as upon various nutrition substrata, have thus far yielded negative results only.

It is of course quite conceivable that the methods which have been used in the search for these hypothetical forms of living matter may not have been appropriate or sufficiently delicate, but the fact remains that none has been found in the many types of material that have been subjected to examination.

If now we consider the filtrable viruses from this viewpoint, the first question that arises is, have we any evidence to show that they are animate, and this in turn raises the question what shall we regard as a criterion or as criteria of life. In as much as the ability to reproduce its kind is perhaps the most striking manifestation of living matter, it may be well to consider this feature in connection with the various viruses. As a matter of fact all viruses with which we are acquainted are capable of producing their peculiar pathogenic effect in series; e.g., if we inoculate a small quantity of chicken plague virus into a healthy animal, this succumbs after a certain length of time, and we find that an infinitesimally small amount of its blood, when injected into a second animal, will kill this, as in the first instance, and so on for generations without limit. The possibility that this serial effect could be due to the injection of a non-reproducing substance, a toxin of such extraordinary potency, for example, that no evidence of its dilution would become evident even after passage through hundreds of animals, need not be considered. There can be no question at all that the virus is reproduced, but it might rightfully be asked whether an increase, in its quantity in the body of the infected animal of necessity involves its reproduction in the ordinarily accepted sense of the term, or whether such an increase in quantity could not be explained on the basis of a new formation not *ex eo ipso,* but on the part of the tissues of the host from material furnished by the host. If such a process could occur then a mere increase in the quantity of matter could

* See papers by F. d'Herelle and by Alexander and Bridges in this volume. *J. A.*

not be viewed as a criterion of life. As a matter of fact there is a steadily increasing amount of evidence to suggest that reproduction of this type can occur. Thus it has been shown that while the injection of the expressed juice of the leaves of certain variegated plants (Abutilon Thompsoni) into the tissues of susceptible species (Abutilon indicum) will not produce the disease of which this type of variegation is an indication, the grafting of a diseased twig onto the susceptible plant will lead to the appearance of the malady in the latter. It is to be noted, moreover, that the disease will not appear in leaves which have fully developed already, but only in those which are to develop in future. The inference is that the principle which causes the variegation is itself not living matter, that is, it is formed in the living diseased leaves, and that its presence here either directly or indirectly alters the metabolism of the cells composing the *Blattanlage* with the resultant production of the same type of material which was responsible for the deficient production of chlorophyll in the first instance. If this interpretation of the possible serial transmission of the disease in question, which is not a filterable virus disease, to be sure, should prove correct, it is conceivable that the same principle may play a rôle in connection with some of the virus diseases proper. In future it will have to be considered in all investigations dealing with the nature of those viruses, at least, which have thus far remained invisible. At the present time this viewpoint is being discussed with particular interest by students of the so-called d'Hérelle phenomenon. This is essentially a transmissible lysis of bacteria which according to d'Hérelle and his supporters is caused by an animate filtrable virus, while others believe that the lytic principle is an inanimate agent, and assume that it is formed by the bacteria themselves, either as the expression of an acquired hereditary metabolic defect, or as the result of the activity of a principle which is liberated during the dissolution of the organisms, and acting upon others, stimulates these to the reproduction of the same substance, and so on.

In connection with mosaic disease of tobacco the inanimate nature of the causative agent has been advocated on repeated occasions. Beijerinck noted the possibility of the transmission of the disease in series, and hence assumed the reproduction of the infective principle; but in as much as he found the bottom layers of a thick agar plate which had been covered with crushed leaves of a diseased plant, infective after the surface had been washed not only with distilled water, but even with strong mercuric chloride solution, he concluded that the principle could not be particulate, but must be present in solution. He accordingly conceived the idea of a *contagium vivum fluidum*. As he could further show that no increase in the quantity of the infective principle took place in vitro, using the juice of healthy plants as culture medium, and that only those leaves of inoculated plants developed the disease in which active cell division was taking place, he not unnaturally concluded that the virus can reproduce itself only when bound to the living protoplasm of the host cell. Regarding the manner in which he conceived reproduction to occur, Beijerinck did not express himself, but he admitted that the idea of a division of metabolizing molecules could not be entertained.

While on first consideration the idea of a *substantia viva* fluid, seems to be repellant, it must be admitted that it can after all not be discarded without better reasons than could be adduced at the present time. However this may be, the fact that Beijerinck could demonstrate the presence of the mosaic disease virus in the lower layers of his agar plates, can after all not be viewed

as an argument against the particulate nature of the virus, as we now know that the size of the infective particle is such that it could readily pass the pores of agar filters having the consistence of the usual agar culture media.

A couple of years following Beijerinck's work, Wood suggested a "physiological" origin of mosaic disease, postulating the presence of excessive quantities of oxidases or an excessive activity of oxidase in the diseased cells. This view, however, could no longer be maintained, when Allard showed a complete lack of correlation between the content on the part of the diseased leaves of oxidases on the one hand, and their infective power, on the other.

If on the basis of the transmissibility in series of the respective diseases which are caused by the filtrable viruses, the animate nature of the latter cannot be regarded as proven, the question suggests itself whether a study of the size of the infective particles in relation to the possible occurrences of nutritive processes may not throw some light on the subject.

While the term filtrable virus of course implies that the size of the infective particle is smaller than the effective pore size of the more commonly employed filters, viz., less than 0.5 μ, we have as yet no knowledge of the actual size of the majority of the viruses. In the case of two typical representatives of the group, however, this has been worked out with some precision by the aid of the method of comparative ultra filtration and using certain colloidal standards as test objects. Andriewsky thus found that the particles of the chicken plague virus were smaller than the colloidal particles of a one per cent solution of hemoglobin, and about the size or a little smaller than those of serum albumin. For the mosaic disease virus Duggar and Karrer found that the infective particles have about the size relations of *fresh* hemoglobin, which they assumed to correspond to a diameter or 30 mμ more or less.*

The chicken plague virus would thus be a little smaller than the mosaic disease virus. The question now arises whether submicroscopic dimensions of such an order would be reconcilable with that minimum of organized structure which one would have to postulate for living matter. MacKendrick and Errera, on the basis of hypothetical protein formulas, calculated out that a coccus having a diameter of 50 mμ could hold in addition to water and salt only one thousand protein molecules, and one with a diameter of 10 $\mu\mu$ only twelve such molecules. Dimensions such as these would be difficult to reconcile with an organized cellular structure of the usual types on the part of the viruses in question, but the nucleated cell is no longer recognized by biologists as the lowest unit of living matter, since differential products such as chromosomes, centrioles and trophoblasts have been discovered which are endowed with the essential attributes of living matter, and which to a certain degree appear to be functionally independent. It is true that the structures mentioned are visible with the microscope, but there is no cogent reason against assuming that yet smaller but autonomous bodies of a similar order may not exist. So long as the viruses can be shown to be composed of a multiple of molecules we may conclude that theoretically such a minute size is in itself not irreconcilable with their animate nature. Andriewsky's conclusion to the contrary in the case of the chicken plague virus was evidently due to the fact that he confused the size of colloidal particles of hemoglobin with the size of hemoglobin molecules. While we could theoretically

* For solutions made up from dried hemoglobin Bechhold in his text book "Die Kolloide in Biologie und Medizin" gives 30-33 $\mu\mu$ as the size of the particles. English translation by Dr. J. G. M. Bullowa. D. Van Nostrand Co., New York.

imagine the reproduction of an animate virus so long as it was composed of several molecules, the idea of dividing and growing living molecules would be unbearable without abandoning the definition of what constitutes a molecule.

While the possibility of producing disease in series can, as we have seen, no longer be regarded as a criterion of the animate nature of the filterable viruses, their minute size thus is in itself no proof to the contrary.

To turn now to other possible criteria of life, we must consider the possibility of cultivating the filtrable viruses on artificial media, and the associated, but not necessarily dependent problem of their microscopic demonstration. It goes without saying, that their animate nature would be proven, if evidence could be adduced to show that an increase in visible bulk or an increase in activity took place in vitro.

It has long been known that peculiar cell inclusions appear quite constantly in lesions that are caused by filtrable viruses of various kinds; familiar examples are the bodies of Henderson and Patterson which are found in connection with molluscum contagiosum, the cell inclusions of Bollinger or poultry pox, the Borrel bodies of sheep pox, the Guarnieri bodies of small pox, the Negri bodies of rabies, the trachoma bodies of v. Prowazek and Halberstaedter, etc. The significance of these cell inclusions has long been in doubt. A number of them have been viewed as protozoa and as the causative agents of the corresponding diseases. On the basis of his studies of the inclusions found in trachoma v. Prowazek evolved his now well known chlamydozoal doctrine, viz., he regarded the cell inclusions in question as being composed of exogenous virus particles surrounded by a reactive substance furnished by the invaded cell. On the whole v. Prowazek's morphological findings have been confirmed and the protozoal nature of these inclusions is no longer upheld. Further studies have then shown, however, that microscopically visible granules corresponding to v. Prowazek's virus particles proper may occur in infective tissues, without apparently being surrounded by a mantle of reactive cellular substance. Lipschuetz accordingly proposed the term strongyloplasms to denote all such microscopically visible virus particles, irrespective of the presence of a mantle. Typical examples are the granules which Borrel first described in sheep pox, elementary (Volpino-Paschen) bodies of small pox and vaccinia, the granules found in molluscum bodies and in the diseased cells of epithelioma contagiosum, etc. All of these granules are of such extremely minute size that they could certainly pass those filters which have been shown permeable for the infective agents of the corresponding diseases. The question, however, remains do these granules actually represent the viruses in question and are they animate. In as much as the granules are invariably found in diseased tissues, inoculation of which will invariably produce the identical diseases, that they can be filtered off from Berkefeld filtrates, for example, by passage through ultra filters, the filtrates from the latter becoming non-infective, while the filtering surface becomes infective and contains granules of the same morphological types and presenting the same resistance to staining processes as the original granules, all these data strongly suggest that they represent the infective agent. The possibility, however, remains that they in turn may be aggregates of the infective agents and that these may after all be inanimate in character. Opposed to this idea is the fact that amongst such granules many diplo and dumbbell forms may be seen, suggesting that actual reproduction of the infective particles is taking

place. These observations furnish perhaps the best proof that we possess of the probable animate nature, of this group of filtrable viruses at any rate. It might be viewed as absolute were it possible to substantiate them by similar findings in cultures in vitro.

The successful cultivation of a number of filtrable viruses has, as a matter of fact been claimed, but on the whole the findings are not altogether convincing. The mere fact that positive inoculation results may be obtained with a "culture" removed even by a number of generations from the original "culture," viz., the tube that was first inoculated with infective material, does not prove by any means that a culture has actually been obtained. Some of the viruses are still active in such extreme dilution that it is quite conceivable that the successful inoculation of an animal after a number of supposed passages may mean nothing more than a passive transfer of a sufficient quantity of the original virus. This criticism would apply more particularly to Marchoux's claim of the successful cultivation of the chicken plague virus.

The mere demonstration of granules in cultures in vitro can certainly not be regarded as proof of cultivation, even though diplo and dumbbell forms may be found. It would certainly have to be shown that the corresponding disease or lesion could be produced by inoculation with such a culture. This proof has not been furnished in connection with the claimed cultivation of the virus of molluscum contagiosum.

The cultivation of the vaccine virus has been claimed on repeated occasions, but if we bear in mind the remarkable viability of this virus outside of the body, it cannot be surprising that successful inoculation results should have been obtained with supposed cultures.

All this work, including that done on the virus of infantile paralysis, requires careful repetition with all possible controls. For the present we cannot say that the cultivation of a single representative of the group has been accomplished without possibility of doubt.

If we accept the visible granules which are found in connection with various virus diseases as the viruses causing the diseases and as probably animate, on the basis of microscopic findings, it would be appropriate to separate this group from the remainder in which the microscopic demonstration of their particulate character has not yet been achieved. Representatives of this group are the virus of measles, of mumps, of dengue, of pappataci fever, of chicken plague, of Rous' infective sarcoma, of Rinderpest, hoof and mouth disease, hog cholera, South African horse sickness, catarrhal fever and heart water disease of sheep, the virus of mosaic disease and the bacteriophage. The fact that these viruses cannot be rendered visible with the optical means which are now at our disposal does not imply, of course, that they are so small as to be actually ultramicroscopic. It has been shown by Zsigmondy that the limit of visibility of colloidal particles is 5 $m\mu$ and we have already drawn attention to the fact that the size of the infective particles of the chicken plague virus, which apparently is one of the smallest of the group, is approximately 30 $m\mu$. If then these particles cannot be seen we may properly assume that this is owing to certain technical difficulties which have not yet been overcome.*

Even though the viruses in question have not been seen with the ultra-

* To be visible, ultramicrons must have a materially different index of refraction from that of the dispersion medium. *J. A.*

microscope, there is evidence to suggest that they may be particulate. The virus of hoof and mouth disease is thus held back by porcelain filters of the denser type, while it readily passes the wider pored forms. The same is true of the virus of hog cholera. The virus of Rous' infective sarcoma of chickens will pass the diatomaceous Berkefeld filters, but is held back by the porcelain Chamberland forms. The virus of chicken plague, and of the mosaic disease of tobacco readily pass the various stone filters and even the wide pored agar and collodion ultra filters, but are held back by the denser forms.

Doerr, however, who is probably the chief exponent of the modern doctrine that inanimate agents may produce infectious diseases, does not view the particulate nature of a virus as opposed to the possibility of its being inanimate. He is one of the group of investigators who look upon the bacteriophage as inanimate, and he believes that the virus type which has been shown to be responsible for the production of the infectious sarcoma of chickens probably belong to this order. As argument in favor of this view he points out that the production of these tumors in the laboratory is more apt to be successful, if at the time of inoculation the cellular elements in the affected area are stimulated to reproduction by the simultaneous injection of some foreign substance, such as diatomaceous earth or the like. He infers that the dividing cells * are influenced more readily by the virus than quiescent cells, and that in this respect the mechanism underlying tumor production of this type is comparable to what we observe in connection with the d'Hérelle phenomenon, where the lysis of the corresponding bacteria takes place only while these are in process of active reproduction. Were the inference which Doerr draws from the similarity between these two types of virus activity a proper one, however, we should also be justified in regarding the mosaic disease virus, the vaccine virus, the epithelioma contagiosum virus and perhaps still others as inanimate, for in connection with these also do we find that active reproduction of susceptible cells is necessary, if not for mere localization of the corresponding viruses, yet at least for their reproduction. On inoculating healthy tobacco plants with the mosaic disease virus we thus find that the disease will appear only in those leaves which at the time of inoculation were in the state of the Anlage, and in their descendants. In the case of the epithelioma contagiosum virus its inoculation into the circulation will be followed by the appearance of lesions only in previously traumatized areas. The vaccine virus, when injected into the circulation of rabbits will become deposited in various parts of the body. but it will multiply only when active cell production is either going on normally, as in the reproduction of glands, or in susceptible tissue which has been injured. While the idea of the inanimate nature of the virus of the mosaic disease of tobacco is perhaps not so repellent, the assumption of the inanimate nature of the vaccine virus would seem to be reconcilable with greater difficulty with certain facts, even other than its appearance under the microscope in particulate form and apparently showing evidence of active division. One of the greatest objections in the writer's opinion would be the wide range of animal susceptibility to this particular virus. It would be difficult to conceive an infective agent of such low specificity on the one hand, as to produce infection of such widely different animals as man, cattle, horses, rabbits and even chickens, when coupled with the production of structurally such highly specific lesions as the skin pustules in the primarily infected animal and the corre-

* See paper by E. E. Just, this volume. *J. A.*

sponding corneal lesions which develop on transfer to rabbits. To account for such a combination of properties on the basis of adaptation on the part of the living organism would be simple, but to endow an inanimate agent with such characteristics would scarcely be warrantable.

To summarize then we may say that the filtrable viruses proper are neither bacteria nor protozoa in the ordinarily accepted sense of these terms, that they are probably all particulate, that some apparently are visible with the microscope, that their animate nature has not been proven, but that indirect evidence suggests that the majority of them are living matter. There is a possibility that some of the viruses may be inanimate, but that this also has not yet been proven beyond a doubt. While the application of colloid chemical studies to this interesting group of disease producing agents may aid in solving the problem of their nature, the little that has been done thus far has not been sufficient to warrant any far-reaching conclusions.

Bacteriophage, A Living Colloidal Micell

By Dr. F. d'Herelle,*

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Most biologists agree that all living matter exists in the colloidal condition, and that all physiological and pathological reactions occurring in living beings are dominated by the laws of colloid chemistry. However, even among the most advanced biologists, there are, I think, few who yet clearly perceive how far the domain of this science actually extends. In what follows, I will be obliged to depend on biological experiments; I will try to be as clear as possible, so that readers unfamiliar with biological methods may follow the line of demonstration.

In 1835 J. Muller formulated what may be called "the theory of cellular protoplasm," which may be epitomized as follows: the unit of living matter is the cell, which is made up of a mass of protoplasm differentiated into cytoplasm and nucleus; all living beings are composed of at least one cell (unicellular organisms); with multicellular beings the organism is formed of a more or less complicated grouping of different cells, the cells of like kind being united into tissues. The cellular theory serves as a basis of biological science. Is it correct, that is, does it agree with the facts? Let us see.

Pasteur it was who definitely proved the importance of microbes as pathogenic agents. But it had long been known that there existed extremely small beings, which could be seen only by means of the microscope; and certain philosophers, first of whom was Malebranche (1674), raised the question as to whether there were not still smaller organisms which the microscope could not reveal. Pasteur, in 1881, in examining the cerebral substance of animals dead of rabies, was unable to find anything resembling a parasitic microbe, and concluded that the pathogenic agent is too small to be seen, even at the highest magnifications.

This experimental idea of an "infravisible" pathogenic agent, was subsequently clarified more and more, and we now know numerous diseases caused by being which have been denominated successively invisible microbes, filterable viruses, ultraviruses, and ultramicrobes. I have proposed for them the generic title *protobes*. Until recent years all that was known of these beings is that they are invisible, either because they are too small, or, as some claimed, because we did not know how to stain them to make them visible, as had been done with the Treponema of syphilis. This view is made somewhat plausible by the fact that their passage through a porcelain filter is rather in the nature of an accident; in reality, non-filtrability is due to adsorption of the virus by the silica or the clay which forms the filter candle. Furthermore, it was not even known whether these invisible pathogenic agents were

* Translated by Jerome Alexander.

living, or if it was a question of products arising from the cellular metabolism of the diseased organism.

In 1917[1] I described the following phenomenon. A few drops of the evacuations of a patient convalescent of bacterial dysentery, were emulsified in 10 cc. of bouillon (pH between 7.2 and 7.8), and the emulsion was filtered through a porcelain candle. One drop of this filtrate when added to 10 cc. of a young, well developed culture of dysentery bacilli (containing from 300 to 400 million bacilli per cc.), caused within four or five hours complete solution of all the bacilli; the culture bouillon which was at first quite turbid, was then perfectly limpid.

If we filter through a porous candle this liquid made limpid by a drop of the filtrate from the evacuations, and then add a drop of this last filtrate to another young, well developed culture of dysentery bacilli, the same phenomenon reappears; after four or five hours the liquid becomes perfectly limpid, because of the solution of all the bacilli. If we now filter this second culture which has become limpid, and add a drop of its filtrate to a third culture of dysentery bacilli, the solution phenomenon is again reproduced. We may thus continue the series indefinitely, and instead of fading out, the phenomenon becomes more marked. After several passages from culture to culture, the addition, to 10 cc. of dysentery bacillus culture, of an amount of filtrate equivalent to 1×10^{-9} cc., suffices to cause, with a few hours, the complete solution of all the bacilli in the culture.

The phenomenon just described is known as *bacteriophagy* and the agent that produces it is *bacteriophage*. The phenomenon, furthermore, is not limited to dysentery, but is a very general one: at the beginning of convalescence from a disease of bacterial origin there appears in the patient's organism a *principle* which has the power of killing and dissolving the pathogenic bacteria. This fact has been demonstrated for diseases as different as bacterial dysentery, typhoid fever, furunculosis, bubonic plague, and cholera, merely to mention human diseases.[2]

I then showed that the bacteriophagic principle is particulate. An outline of the proof follows.

Take 10 cc. of a well developed, that is, quite cloudy culture of cholera vibrios, for example,* add 1×10^{-6} cc. of a filtrate containing a bacteriophagic principle active against the cholera vibrio, and then mix well. Take six Petri dishes containing nutritive agar, and spread on the surface of each dish 0.1 cc. of the mixture. After incubation, we will see that the surface of the nutritive agar in each dish is covered with a layer of cholera vibrios speckled with perfectly circular discs, where the gelose is bare. In one experiment, the average number of discs per dish was thirty.*

This experiment suggests the hypothesis that the active principle exists in the form of corpuscles, and that each clear disc on the gelose represents a spot where there has been deposited during the spreading, one of these corpuscles, which had been developed at the expense of the surrounding vibrios and formed a colony. It may, besides, be readily shown that each drop includes a bacteriophagic principle which does not exist outside the drops.

[1] F. d'Hérelle, *Compt. rend.*, **165**, 373 (1917).
[2] F. d'Hérelle, "Bacteriophage, its Rôle in Immunity," Williams & Willkins, Baltimore, 1922.
* Any bacterial culture at all may be taken, and a filtrate containing a bacteriophagic principle active against the same species of bacteria may be used for the experiment. I have here chosen experiments made with cholera vibrios.
* This number varies from one experiment to another, for the lytic power varies from one case to another.

To verify this hypothesis, the following experiment may be made. Take the same filtrate used in the disc experiment, and determine the limit of its action; that is, the smallest possible quantity capable of dissolving a culture of cholera vibrios. It will be found that if we mix 10 cc. of cholera vibrio culture with 1×10^{-9} cc. of filtrate, clarification is complete within several hours, just as complete as if we had added several drops; on the contrary, 1×10^{-10} cc. produces no clarification, the culture continuing indefinitely to look like a pure culture. Now take 10 cc. of bouillon, add 1×10^{-9} cc. of filtrate, agitate to ensure thorough mixing, and distribute the mixture in ten culture tubes of cholera vibrios. After incubation, three of these cultures will be found perfectly limpid, but the seven others will be found just as cloudy as the controls; and check experiments will show that no trace of the bacteriophagic principle exists in the seven tubes.

These experiments prove that the bacteriophagic principle is *particulate*, that is, it exists in the form of corpuscles suspended in the containing liquid. Those cultures which received a bacteriophage corpuscle exhibit the phenomenon of bacteriophagy. The ones which received a quantity of filtrate containing no corpuscle, remained normal. I would remark that these experiments enable us to estimate the number of bacteriophage corpuscles in a liquid. In the filtrate used in the preceding experiments, there were 3 corpuscles in 1×10^{-9} cc., that is, 3 billion per cc. of the non-diluted filtrate.[3]

I then showed that bacteriophage corpuscles would pass not only porcelain filters, but also ultrafilters of collodion; and that each corpuscle probably is slightly smaller than a molecule of serum globulin, or rather, than a micell of serum globulin. For experiments with filter membranes of different porosity show that bacteriophage corpuscles readily pass through membranes permitting the passage of globulin, but are held back by those impermeable to globulins.[4] Prausnitz, using the same methods, found that bacteriophage corpuscles approximated in size particles of colloidal silver of known size, that is 20 millimicrons in diameter;[5] Bechhold[6] estimates a diameter of 30 millimicrons.*

All this shows that the bacteriophage corpuscle is a simple colloidal *micell*, and that this micell has the power of reproducing itself at the expense of living bacteria. What is the biological nature of this micell? The fact that it reproduces itself in the course of its action is not sufficient of itself to prove its living nature, for the objection may be raised, and was raised,† that the corpuscle may be a product derived from the bacteria which undergo solution, although in reality this latter hypothesis is unable to explain all phenomena involved in bacteriophagy. This however is of no importance, since we may prove directly the living nature of the bacteriophage corpuscle.

The *essential* and *satisfactory* proof of the living nature of a thing is to find out if it has the general attributes of life. What are the fundamental attributes of life? They are *autonomy, assimilation,* and *adaptation.*

Autonomy involves the possession by a being of peculiar characteristics, different from those of surrounding beings. Proof of autonomy is easy,

[3] F. d'Herelle, *Compt. rend. Soc. Biol.*, **83**, 247 (1920).
[4] F. d'Herelle, *Compt. rend. Soc. Biol.*, **81**, 1160 (1918).
[5] K. Prausnitz, *Centralbl. Bakt. I. O.*, **89**, 187 (1922).
[6] H. Bechhold, *Z. Hyg. Infektionskrankheiten*, **105**, 601 (1926).
* Since *m* is the accepted abbreviation for one thousandth and μ for one millionth, the abbreviation for millimicron should be mμ, and not $\mu\mu$, as has been quite common usage. *J. A.*
† Readers particularly interested in this question, will find an outline of the various theories in "Bacteriophage and its Behavior," by F. d'Herelle. Williams & Willkins, Baltimore, **1926**.

and is even implied in the idea of saprophytes, which possess the power of assimilating dead substances. On the other hand, proof of autonomy is fundamental when the being in question is a strict parasite whose power of assimilation can be exercised only on living substances, as is the case with bacteriophage. True assimilation, or chemical assimilation, or assimilation in a heterogeneous medium, consists in transforming substances which are chemically heterogeneous with respect to the being having this power, into substances chemically homogeneous with respect to it. Adaptation is the power possessed by a living thing of being able to change its characteristics in such a way that it may continue to exercise its assimilative power, in spite of changes in the medium in which it is compelled to live.

These are the fundamental properties of life. All beings having them are living; those which do not have them, or no longer have them, are inanimate.

From the fact that bacteriophage reproduces itself in the course of action, it is necessarily either *a living being* of itself, or else a product liberated by the bacteria which undergo solution; in the latter case, it would be *an autolytic enzyme*. We will now give some experiments which will enable us to decide which of these two hypotheses is correct. We will begin with proofs of autonomy.

A certain bacteriophage *h*, attacks and dissolves all strains of golden, yellow, and white staphylococci; it therefore possesses a "polyvalent" character. Another bacteriophage *v*, is strictly "monovalent," and attacks only one strain, staphylococcus V, its action on any other staphylococcus being insignificant. If bacteriophage is an autolytic enzyme produced by the bacteria, the characteristics of enzyme-bacteriophage *v* must depend only on the characteristics of staphylococcus V which secretes it; the "monovalent" character, among others, is impressed by staphylococcus V.

If, however, the characteristics of "monovalence" and "polyvalence" do not depend on the staphylococcus at whose expense they are reproduced, then these are *personal* characters belonging to the corpuscles themselves, independent of the medium in which they reproduce themselves; in this latter case, they would be autonomous beings.

We can easily prove which of these two alternatives is true, for bacteriophage *h* is polyvalent; it can dissolve staphylococcus V and reproduce itself at the expense of this organism. If we make a long series of passages of bacteriophage *h* through cultures of staphylococcus V, we will see that after a number of such passages bacteriophage *h* has kept its individuality and characteristics, especially its polyvalent character. The monovalent characteristic of bacteriophage *v* does not, therefore, depend on staphylococcus V at whose expense it reproduces itself. The properties of each bacteriophage are, therefore, independent of the characteristics of the bacteria, and each bacteriophage is, therefore, an autonomous being, independent of the bacterium which is *heterogeneous* with respect to it.

But we have previously shown that in the course of action, bacteriophage corpuscles multiply in the proportion of one to several billions; the mass of bacteriophage therefore increases, and this increase can be only at the expense of the substance of the bacteria. Since we have just seen that the bacteriophage corpuscle is an autonomous being, it must use for its development bacterial substance heterogeneous with respect to itself. Bacteriophage, there-

fore, possesses the power of assimilation in a heterogeneous medium, which is one of the fundamental characteristics of life.*

There remains the third fundamental property of living beings, the power of adaptation.

Most bacteriophages that we can isolate in nature do not cause, *in vitro,* the phenomenon of bacteriophagy in media whose pH is less than 6.8, the optimum being generally about 7.8. By taking a bacteriophage active against dysentery bacilli, which caused bacteriophagy only in alkaline media, and by

TABLE I.

	Number of Bacteriophage Particles	
	After 24 Hrs. Contact with Sublimate	After 48 Hrs. · Contact
Bacteriophage which has been subjected to 9 passages through media containing increasing amounts of sublimate.		
(a) placed in a 1-10,000 solution of sublimate	about 2,000	370
(b) in physiologic salt solution..............	about 2,000	410
Same bacteriophage, *not adapted*	0	0
(a) placed in a 1-10,000 solution of sublimate	454	174
(b) in physiologic salt solution..............	0	0

passing it successively through cultures of dysentery bacilli in which the pH was slightly lowered in each passage, I was able to produce bacteriophagy in definitely acid media, pH 6.4.[7] Asheshov[8] and Schurman[9] have also reported conclusive experiments to this effect. Prausnitz[10] has been able to adapt bacteriophage to chloramine, to corrosive sublimate, and to phenol. The two following tables give a résumé of his experiments on sublimate and phenol.

TABLE II.

	Number of Corpuscles: Suspension of Bacteriophage Added to an Equal Quantity of		Loss per 100
	Phenol 15-1000	Distilled Water	
Bacteriophage which has been submitted to 22 passages through phenol........	632	768	18
Same bacteriophage, passed through media without phenol..............	0	578	100

I doubt if a more formal proof of power of adaptation could be given for any living thing.

The bacteriophage corpuscle is, then, an autonomous being which has to a very high degree the power of adapting itself to adverse conditions in the

* In the book previously cited, I have given ten different proofs of the autonomy of bacteriophage. Similarly, I have given eight different experimental proofs of adaptation based on my own experiments and those of other authors.

[7] F. d'Herelle, "Der Bakteriophage," p. 113, Vieweg & Sohn, Braunschweig, 1922.
[8] Asheshov, *Compt. rend. Soc. Biol.*, **87**, 1343 (1922).
[9] C. J. Schurman, *Centralbl. Bakt. I. O.*, **93**, 148 (1924).
[10] Prausnitz & Firle, *Centralbl. Bakt. I. O.*, **93**, 148 (1924).

medium. Beyond any question, it is a living being. On the other hand, it is a fact that this unquestionably living being is made of a single micell. Furthermore, bacteriophage is not the sole "micellary" being; the active virus of rabies, of vaccine, of smallpox, of encephalitis lethargica, of bird pest, of plant mosaics, all pass through ultrafilters. Levaditi[11] has shown that with rabies, vaccine, and encephalitis lethargica, the dimensions are exactly similar to those of bacteriophage; for when one of them passes, all the others pass, and when one is retained, so are the others. The same is the case with the virus of chicken sarcoma, as I have proved. It is possible to prove the living nature only with bacteriophage, but all these beings are so similar that it is extremely probable that they are all living beings. I have proposed the name *protobes* for these beings made of *a single micell*.

There are, therefore, living beings possessing the same characteristics as all other known living beings, but which are formed of a single colloidal micell, and this suffices to prove that the so-called cellular theory, which really serves as the basis of all biology, is certainly erroneous. The smallest amount of *autonomous* living matter is *not the cell, but the micell*. And this statement necessitates a complete modification of existing biological concepts; I can not here go into the question in detail but will simply point out some of the consequences of what may be called the "micellular theory" of life.

The cell is an organism already very complicated, and it is difficult to understand how a cell could have appeared on earth, already formed: in the cellular hypothesis, the origin of life remains a mystery. This is no longer the case if much simpler living beings exist, which are formed of a simple micell. I do not wish to say (as some authors would have me say) that bacteriophage is the primative being, for this latter could evidently not be a parasite, but must be a micellary being capable of building up organic material of all kinds by using mineral substances. I have indicated[12] that such beings exist in certain sulfurous mineral springs. Owing to circumstances beyond my control, I have been obliged to cease experiments on this subject, but propose to take them up again as soon as I find opportunity.*

In any event, the existence of micellular beings shows that living things are not divided into mono- and multicellular beings, as existing classification has it, but that there are three grand divisions, perhaps four. At the bottom are the protobes, composed of a single protoplasmic micell. Above the protobes, come beings made up of aggregates of micells, each micell being capable of reforming the aggregate, and each aggregate constituting a *non-differentiated* cell. These aggregated beings are bacteria which are at the bottom of the vegetable kingdom, and spirochetes which are at the bottom of the animal kingdom. Higher up are true cellular beings, formed of cells which are differentiated into nucleus and cytoplasm; that is, beings included in the existing classification.

The existence of autonomous living micells also suggests that among multicellular beings, the fundamental living unit is not the cell, but the micell. Micellular beings would thus be of the first order, cells of the second order, and multicellular beings of the third order. Among these last mentioned, the basic unit would be the micell: a cell would be an organism formed by the union of micells of different types, the micells of each type being grouped

[11] Levaditi & Nicolau, *Compt. rend Soc. Biol.*, **88**, 66 (1923).
[12] F. d'Hérelle, "Immunity in Natural Infectious Disease," Williams & Willkins, **1924**.
* This paper of Dr. d'Hérelle was sent from India, where he was studying cholera, bubonic plague, etc. *J. A.*

into nucleus, cytoplasm, etc. Each micell would have its own power of assimilation, that is, its individual metabolism, the cellular metabolism being the sum total of the micellular metabolisms.

In any event, the proof of the existence of living beings formed of a single colloidal micell, suffices to prove that the theory of cellular protoplasm, which forms the basis of present-day biology, is incorrect. It furthermore shows that colloid chemistry is the basis of even life itself, and that at some doubtless still distant future, this science will solve the nature of life. Without looking so far ahead, I believe that in the near future the whole of microbiology will become a chapter in colloid chemistry. Indeed, micells, whether living or not, all of the reactions occurring in them, and their entire behavior—are they not all governed by the same laws? Because a micell is living, will the laws governing surface tension, for example, differ from what they would be with a non-living micell? Certainly not; and the moment when the laws governing the behavior of the micell are always and everywhere the same, be it living or not, microbiology is in reality the science of living colloids.

The Colloidal Behavior of Bacteria

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I. Introduction

During the past decade it has become increasingly clear that many phenomena evidenced by living cells in general and by the bacterial cell in particular are as clearly associated with physicochemical as with biochemical factors in metabolism. More particularly, it appears that many of the properties exhibited by bacteria suspended in nutrient or in non-nutrient menstrua are determined by mechanisms which are not directly associated with viability, mechanisms which commonly play significant rôles in the determination of the behavior of matter in colloidal state.

If we accept the definition of a colloidal solution provided by Burton,[1] namely:

Generally, a colloidal solution may be defined as a suspension, in a liquid medium, of fine particles which may be graded down from those of microscopic to those of molecular dimensions; these particles may be either homogeneous matter, solid or liquid, or solutions themselves of a small percentage of the medium in an otherwise homogeneous complex. Such solutions may be prepared in almost numberless ways, and, in their properties, may betray variations as numerous as the methods of preparation. The one property common to all such solutions is that the suspended matter will remain almost indefinitely in suspension in the liquid, generally in spite of rather wide variations in temperature and pressure; the natural tendency to settle due to gravity is overbalanced by some other force tending to keep the small masses in suspension.

we come to the conclusion from *a priori* considerations that bacteria in suspension in a fluid will behave, in many respects, like colloids. Although all of the common bacteria are easily discernible under the microscope, many of the smaller ones are almost precisely at the limit of visibility unless the ultramicroscope is utilized. An average diameter for the smaller, spherical or slightly cylindrical bacteria is 1 μ. Suspensions of these microörganisms in water or in dilute salt or peptone solutions are more or less indefinitely stable, sedimentation occurring commonly only very slowly. Such suspensions display the usual properties of a solution in which the solute is a distinct dispersed phase in a solvent which is a defined dispersing phase. The discontinuity of the phases is as sharply defined as in so-called true colloidal solutions. That the dispersed phase (bacteria) is not in itself homogeneous complicates but does not render impossible the analogy.

A micrococcus with a diameter of 1 μ may have a surface area not less than 60,000 sq. cm. per gram of mass. Hence, it may be expected to show many of the properties of matter in the colloidal state. We may review here only briefly some of the accumulating evidence which indicates that such

[1] Burton, E. F., "The physical properties of colloidal solutions," London, 1921.

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properties bear significant relations to the biological characteristics of the cell which are sometimes assumed to be dependent upon viability.

II. Some General Properties of Bacterial Suspensions

That bacteria show Brownian movement has been known since the days of the early microscopists. The kinetic theory has provided a satisfactory explanation of the origin of this movement and of the mechanism whereby it serves to overcome the tendency for gravitational precipitation. With bacteria, as with typical colloids, the Brownian movement may be inhibited or abolished by the addition of electrolytes; and agglomeration and precipitation commonly follow with bacteria as with ordinary colloids.

The optical properties of bacterial suspensions have not been extensively investigated. The refractive index of the bacteria is apparently not very great. In hanging drop preparations in water they are clearly visible under the microscope as gray particles; in 1 per cent NaCl solutions ($D_{15} = 1.34$) they are still visible; in melted phenol ($D_{15} = 1.55$) they are invisible.[2] The spores of bacteria and the "cell membrane" of vegetative forms as well as of spores show higher refractive capacities than are displayed by the cytoplasm. The "halo" which is visible about any bacterium is apparently optically empty and is itself considered an artefact resulting from the reflection of light waves from the surface of the cell or from the interphase between the thin layer of fluid contiguous to the cell (and which behaves as though it were an integral part of the cell) and the layers of menstruum immediately adjacent. The membranes (or surface regions) of certain bacteria appear to be anisotropic.[3] The Tyndall effect is, of course, easily demonstrated with dilute bacterial suspensions.

The conductivity of bacterial suspensions was measured by Thornton[4] by a method which, according to this author, depends upon the principle that in an electrical field the microörganisms are oriented in a solution of lower conductivity than the bacteria themselves, but not in a solution of the same conductivity. To determine the electrical conductivity of the cells his procedure was to increase the concentration of salt in the menstruum progressively until the bacteria fail to show orientation. Thornton concluded that the electrical conductivity of living bacteria is usually greater than that of the medium in which they grow. It appears questionable that Thornton was measuring a property of bacteria determined by conductivity. His observations are more probably concerned with cataphoresis and with the effects of electrolytes upon cataphoretic potentials. Shearer[5] found from direct measurements of electrical conductivity that in NaCl solutions the electrical resistance of bacteria falls to zero in 30 to 40 minutes. With meningococci or colon bacilli, salts with monovalent cations produce a rapid fall in resistance which is at first reversible and which need not involve the death of the bacteria. On the other hand, acids and most salts with bivalent cations produce a slight increase in resistance which is followed by a fall which is irreversible and which is correlated with the death of the cell.[6]

More recently, Green and Larson[7] have failed to confirm the assertion

[2] Angerer, K. v., *Arch. Hyg*, 93 (1923).
[3] Amann, *Centr. Bakt.*, I, Orig., 13 (1893).
[4] Thornton, W. M., *Proc. Roy. Soc. (Lond.)*, 85, **B**, 331 (1912).
[5] Shearer, C., *Proc. Camb. Phil. Soc*. 263 (1919); *J Hyg*, 18, 337 (1919-20).
[6] Vide criticisms of Shearer's work by Falk, I. S., *Abstr Ba t* 7, 99 (1923).
[7] Green, R. G and Larson, W P., *J Inf Dis*, 30, 550 (1922)

of Shearer that the electrical resistance of *Bact. coli* falls to zero after heating. (Experiments conducted by Holland in Professor Winslow's laboratory at the Yale Medical School had likewise failed to confirm this observation of Shearer.) Green and Larson's findings appear to contradict those of Osterhout and others that when cells die their resistance to the passage of an electrical current becomes zero. Chambers [8] has called attention to a finding which is probably of general biological interest, that the permeability of the surface is not the same as that of the interior of the cell.[9]

III. The Viscosity of Bacterial Suspensions

The colloidal behavior of bacteria can be nicely demonstrated from measurements of viscosity upon bacterial suspensions. The data presented here are taken from a paper by Falk and Harrison [10] on the influence of hydrogen ion concentration upon the viscosity of washed and unwashed suspensions of *Bacterium coli*. In Figure 1 the effects of pH upon viscosity are presented for suspensions containing 5, 10, 17 and 50 billions of bacteria per cc. of suspension.

It appears from the curves in Figure 1 that viscosity increases with increasing concentrations of bacteria and that the effect of pH is significant only in zones near pH $= 3.8$ and pH $= 13.0$. It is interesting to note that in these zones of pH the suspensions show agglutination ("coagulation").

Since the work of Michaelis [10a] it has been known that bacteria undergo spontaneous agglutination at suitable acidities. From more recent investigations [11] it has become clear that the zone of acid agglutination centers about the acid isoelectric point of the bacteria. That bacteria will also manifest an *alkaline* isoelectric point was predicted by Shaughnessy and Falk [12] from experimental findings on the buffering capacities of bacteria at various H-ion concentrations and has been demonstrated experimentally by Winslow, Falk and Caulfield and by Winslow and Shaughnessy.[13] Our findings in the course of viscosity measurements also show the expected agglutination of bacteria in the zone of the alkaline isoelectric point. We propose to show the significance of changes in the state of dispersion of the bacteria in an analysis of the effects of [H⁺] upon the viscosity of bacterial suspensions.

From kinetic considerations Einstein [14] derived a formula according to which the increased viscosity of a fluid which follows upon the introduction of a dispersed phase into it is directly proportional to the relative volume occupied by the dispersed phase. Thus:

$$\eta = \eta_0 \, (1 + k \, \phi) \tag{1}$$

when η and η_0 are the viscosities of the colloidal suspension and of the dispersion medium respectively, k is a constant to which Einstein ascribed the value 2.5 and ϕ is the relative volume occupied by the dispersed particles. According to Einstein, the limiting values of the equation are that ϕ be rela-

[8] Chambers, R., *Proc. Soc. Exp. Biol. Med.*, **20**, 72 (1922).
[9] For further discussion of general colloidal characteristics of bacteria, cf. Falk, *op. cit.* The behavior of bacteria in an electrical field is treated in Vol II, p 115 of this volume
[10] Falk, I. S. and Harrison, R. W., *J. Bact*, **11**, 97 (1926).
[10a] Michaelis, L., *Deut. med Wochenschr*, **37**, 969 (1911).
[11] *Cf.* Northrop, J. H. and DeKruif, P. H, *J. Gen. Physiol.*, **4**, 639 (1922)
[12] Shaughnessy, H. J. and Falk, I. S., *J. Bact.*, **9**, 559 (1924).
[13] Winslow, C.-E. A., Falk, I. S. and Caulfield, M. F., *J. Gen. Physiol.*, **6**, 177 (1923). Winslow, C.-E. A. and Shaughnessy, H. J., *J. Gen. Physiol.*, **6**, 697 (1924).
[14] Einstein, A., *Ann. Physik.*, **19**, 289 (1906); **84**, 591 (1911).
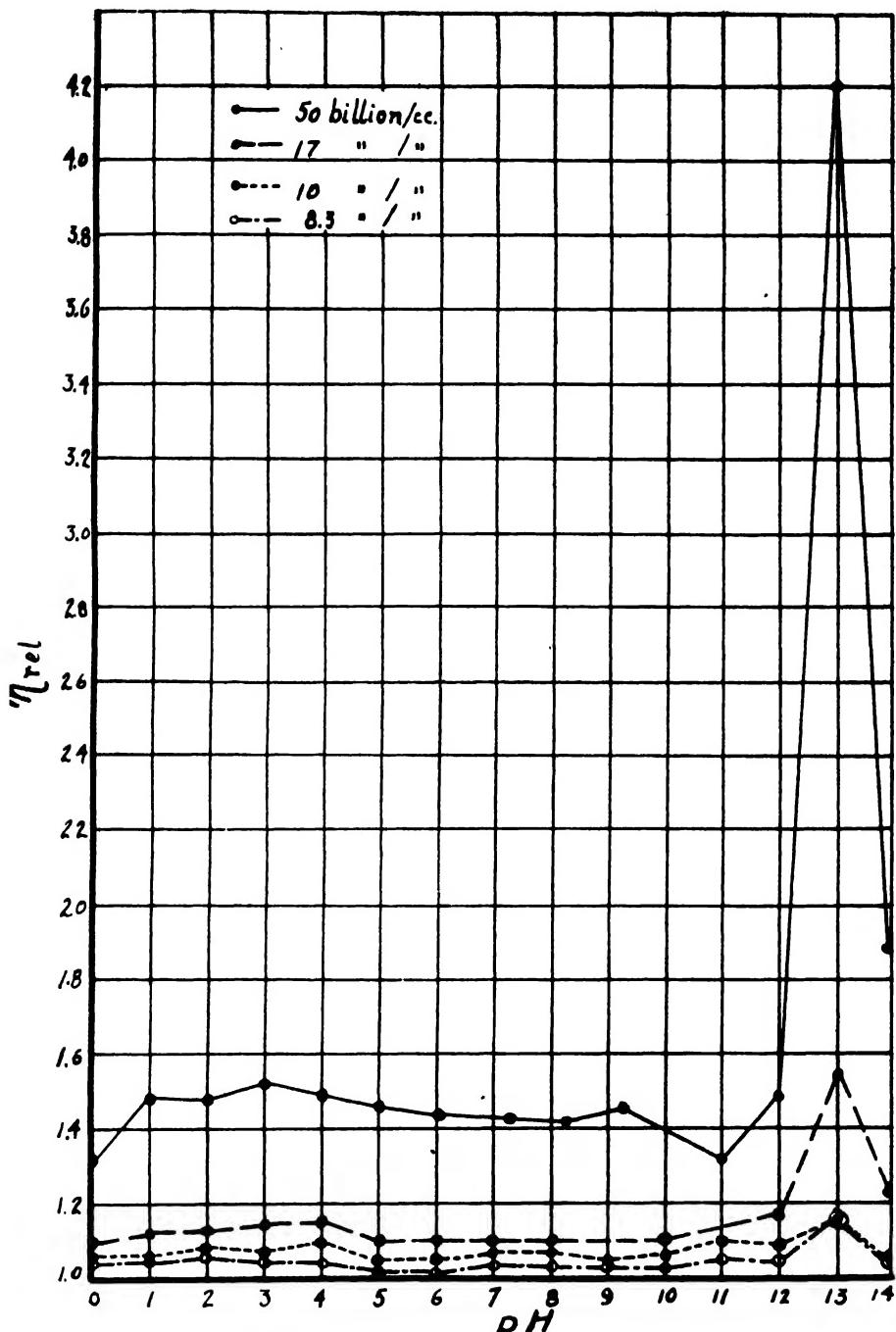
FIG. 1.—The influence of hydrogen-ion concentration upon the viscosity of bacterial suspensions of several concentrations.

tively small and considerably less than 0.5 and that the particles be approximately smooth, non-deformable spheres. The values of k, as determined by many workers, have commonly departed from Einstein's value (2.5) and have ranged from 1 to 4.75 (*cf. Hatschek*).[15] Hatschek first proposed to replace Einstein's constant by the value 4.5. Later he derived the following equation:

$$\eta = \frac{\eta_0}{1 - \phi^{1/3}} \tag{2}$$

His conclusions have been criticized by numerous workers whose experimental findings demonstrate that the constant is not really constant, but is variable according to the nature of the solution being studied.[16]

Smoluchowski[17] has attempted to derive a generally applicable equation which would account for the increases of viscosity commonly observed upon the coagulation of a colloidal solution as well as for its normal viscosity. He arrived at the conclusion that the viscosity is increased by the electrical charge on the particles and that upon coagulation the viscosity must be increased at least 1.35 times if the particles remain even approximately spherical, because particles in approximate contact will appear to have increased in volume by a volume of adherent water or other dispersing medium). Thus:
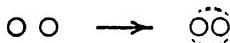
On these grounds Smoluchowski considered that upon coagulation the *apparent* volume occupied by the dispersed phase has increased although the *actual* volume has not changed.

More recently, Arrhenius[18] has derived from Einstein's equation a logarithmic formula which has been widely used to described the viscosity of a colloidal solution:

$$\log \eta - \log \eta_0 = \theta \phi \tag{3}$$

where η, η_0 and ϕ have the same significance as in (1) and (2) above, and θ is a constant.

Loeb[19] has found in his studies on the colloidal behavior of proteins that membrane potentials, osmotic pressure, swelling of gels and viscosity are dependent upon membrane equilibria of the type described by Donnan.[20] With respect to the viscosities of protein solutions his analysis may be considered, briefly, that the viscosity of a colloidal suspension (gelatin, albumin, etc., in water) is a function of water imbibition and swelling; that swelling is a function of the Donnan equilibrium and is dependent upon the degree of ionization of the suspended ampholyte. Hence, viscosity is at a minimum at the isoelectric point and rises to maximal values at [H+] above or below the value at this point. The addition of acid or alkali beyond the concentrations at which these maxima obtain causes diminutions in the viscosity.

[15] Hatschek, E., *Koll. Z.*, **7**, 301 (1910); **8**, 34 (1911); **11**, 280 (1912); **12**, 238 (1913). Also, First Rept. on Coll. Chem., Brit. Assn. Adv. Sci., 2 (1917).
[16] In our later references to "Hatschek's equation" it is his modification of Einstein's equation, using the constant 4.5, to which we refer. Hatschek's own equation which involves the use of the cube root of the relative volume of the dispersed phase ($\phi^{1/3}$) we have found does not even approximate the experimental values.
[17] Smoluchowski, M., *Koll. Z.*, **18**, 190 (1916).
[18] Arrhenius, S., *Medd. Vetenskapakad. Nobelinst.*, 3 (1917).
[19] Loeb, J., "Proteins and the theory of colloidal behavior." New York, 292 pp., 1924
[20] Donnan, F. G., *Z. Elektrochem.*, **17**, 572 (1911).

Loeb has presented extensive experimental evidence which professes to demonstrate the validity of these hypotheses.

In Figure 2 is illustrated the effect of pH upon viscosity of bacterial suspensions (concentrations 1, 3.5, 5, 8.3, 10, 17 and 50 billions per cc. combined). In this figure is also included (by a dotted line) a curve representing a hypothetical extrapolation of Loeb's findings with gelatin. This hypothetical curve is based upon the experimental findings reported by Loeb at pH values in the range 1.0-6.0 and upon the assumptions: (1) that in more alkaline zones the curves are continuous with those in acid zones; and, (2) that the discovery of a second (alkaline) isoelectric point for bacteria at or near pH = 13.0 would demand the occurrence of a second minimum in the viscosity
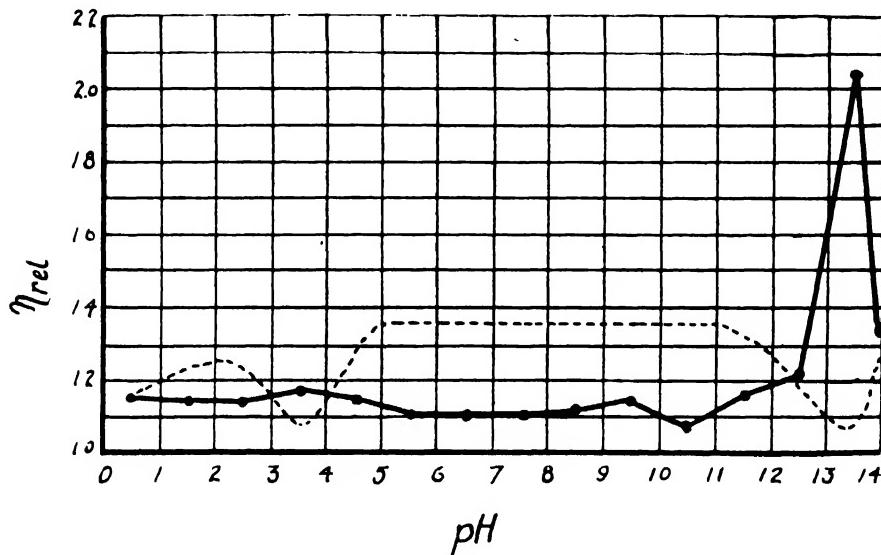
Fig. 2.—The relations between viscosity and hydrogen-ion concentration. The solid line is an average from all experiments, disregarding the different concentrations of bacteria The dotted line is a hypothetical curve based on an extrapolation of Loeb's findings with gelatin (disregarding ordinates).

curve. This hypothetical curve is introduced merely to emphasize our regular observation of a maximum instead of a *minimum* viscosity value at or near an isoelectric point.

In connection with the comparison made between our findings and Loeb's (extrapolated) it is significant to recall that Loeb's viscosity minima with gelatin disappear with time. Thus, he found that the viscosity ratio for 2 per cent gelatin sulfate solution increased from 3 to 7 after standing near the isoelectric point for 10 to 15 minutes [21]

In Tables I-IV and in Figures 3, 4, 5 and 6 are presented the data and a series of curves in which is tested the applicability to bacterial suspensions of the equations presented above. The relative volume (ϕ) occupied by the bacteria was calculated for each concentration from average values of the length and diameter of *Bact. coli,* considering the bacillus a smooth right cylin-

[21] Some years ago, Gokun (*Z   Chem. Ind. Koll.,* **3**, 84 (1908)) reported similarly that the minima in viscosity curves for gelatin + electrolytes disappear after the lapse of several hours.

der $(2.5 \mu \times 0.5 \mu)$. The value of θ was calculated in each case from the observed values of η, η_o and ϕ when the concentration was 5 billions of bacteria per cc. With this calculated value of θ the other values of η_{rel} were calculated.

TABLE I. *Relative Viscosities Measured and Calculated from the Theoretical Equations of Arrhenius, Einstein and Hatschek.*

Concentration (billions per cc.)	η (obs.) Average	η_o (obs.) Average	η Relative $(2) \div (3)$	Volume of Bacteria, (ϕ)	η Relative—Calculated by:		
					Arrhenius' Equation $(\theta = 8.0)$ (calc.)	Einstein's Equation $(K = 2.5)$	Hatschek's Equation* $(K = 4.5)$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
5	90.1	86.1	1.046	0.00246 cc.	1.046	1.006	1.011
10	93.4	86.5	1.079	.00491 cc.	1.096	1.012	1.022
17	99.4	85.8	1.158	.00835 cc.	1.166	1.021	1.038
50	136.9	84.9	1.612	.02460 cc.	1.573	1.062	1.111

* By "Hatschek's equation" in this and subsequent tables we refer to Hatschek's modification of Einstein's equation. Using Hatschek's equation $\eta = \dfrac{No}{1 - \phi^{\frac{1}{3}}}$ we obtain calculated values so far from the measured values of η_{rel} that they are not included in the Tables. Thus, for the data in this Table the calculated values of η_{rel} are 0.865; 1.205; 1.255 and 1.410.
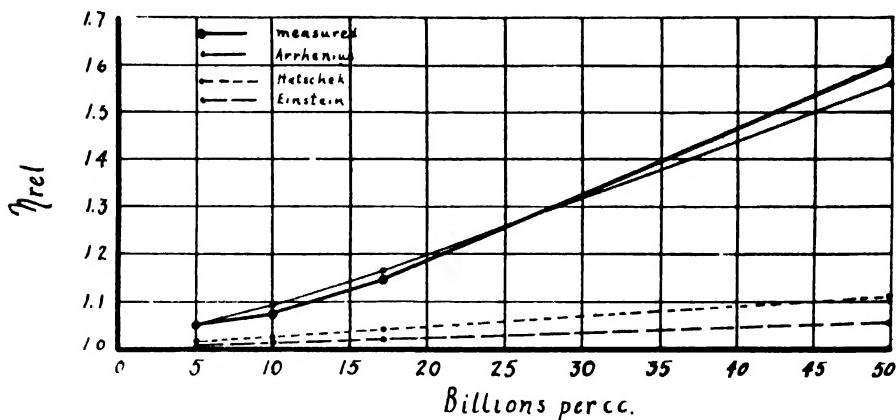
FIG. 3.—The relations between bacterial concentration and viscosity (measured and calculated), all pH values averaged.

TABLE II. *Relative Viscosity (Measured and Calculated) at pH = 6 to pH = 8.*

Concentration (billions per cc.)	η (obs.) Average	η_o (obs.) Average	η Relative $(2) - (3)$	Volume of Bacteria, (ϕ)	η Relative—Calculated by:		
					Arrhenius' Equation $(\theta = 6.0)$	Einstein's Equation $(K = 2.5)$	Hatschek's Equation $(K = 4.5)$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
5	86.0	84.4	1.019	0.00246 cc.	1.035	1.006	1.011
10	91.6	87.0	1.053	.00491 cc.	1.070	1.012	1.022
17	97.2	88.0	1.104	.00835 cc.	1.122	1.021	1.038
50	115.7	81.2	1.425	.02460 cc.	1.405	1.062	1.111
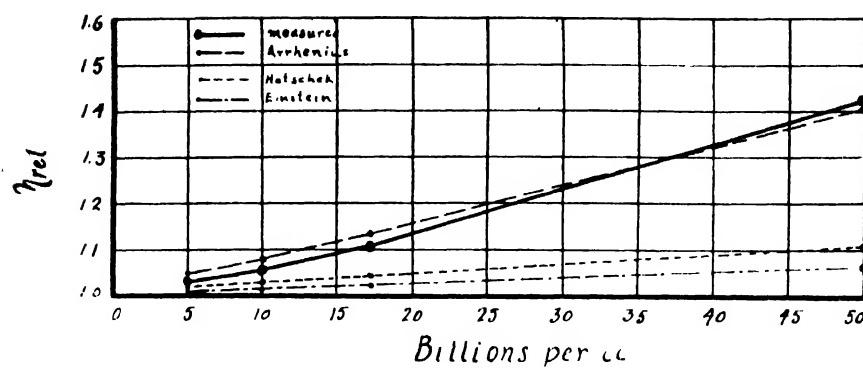
Fig. 4.—The relations between bacterial concentration and viscosity (measured and calculated), pH = 6 to pH = 8.

TABLE III. *Relative Viscosity (Measured and Calculated) at pH = 3 to pH = 4.*

Concentration (billions per cc.) (1)	η (obs.) (average) (2)	η_o (obs.) (average) (3)	η Relative (2) ÷ (3) (4)	Volume of Bacteria, (ϕ) (5)	η Relative Calculated by.			
					Arrhenius' Equation		Einstein's Equation ($K = 2.5$)	Hatschek's Equation ($K = 4.5$)
					($\theta = 6.0$) (6)	($\theta = 7.2$) (7)	(8)	(9)
5	86.7	85.5	1.014	0.00246 cc.	1.035	1.042	1.006	1.001
10	92.4	85.7	1.078	.00491 cc.	1.070	1.085	1.012	1.022
17	100.1	86.9	1.152	.00835 cc.	1.122	1.148	1.021	1.038
50	128.6	85.4	1.506	.02460 cc.	1.405	1.504	1.062	1.111
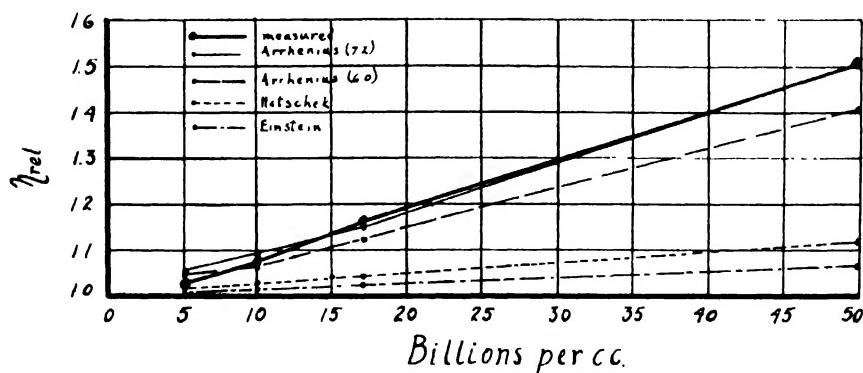
Fig. 5.—The relations between bacterial concentration and viscosity (measured and calculated), pH = 3 to pH = 4.

TABLE IV. *Relative Viscosity (Measured and Calculated) at pH = 13.*

Concentration (billions per cc.)	η (obs.)	η less η of Alk. (obs.)	η_0 (obs.)	η Relative (3)÷(4)	Volume of Bacteria, (ϕ)	η Relative—Calculated by:			
						Arrhenius' Equation		Einstein's Equation ($K = 2.5$)	Hatschek's Equation ($K = 4.5$)
						($\theta = 6.0$)	($\theta = 23.6$)		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
5	99.1	97.1	82.4	1.188	0.00246 cc.	1.035	1.143	1.006	1.011
10	110.6	108.5	92.8	1 169	.00491 cc.	1.070	1.306	1.012	1.022
17	134.6	132.8	84.2	1.575	.00835 cc.	1.122	1.574	1.021	1.038
50	357.6	350.7	83.8	4 185	.02460 cc.	1.405	3.807	1.062	1.111
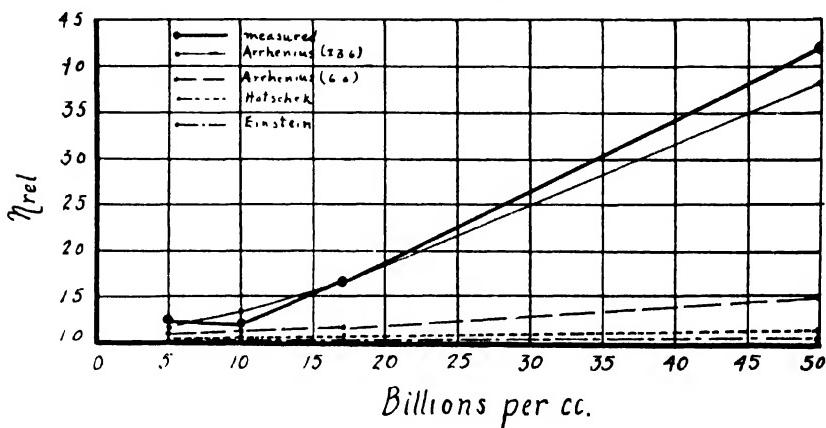
FIG 6.—The relations between bacterial concentration and viscosity (measured and calculated) at pH = 13.

The data and the curves indicate that the equation of Arrhenius is clearly applicable to describe the effect of concentration upon the viscosity of bacterial suspensions. To make the equation conform to data obtained at various pH values we have followed the usual procedure of calculating values for θ for each set of curves. In our opinion, this is not a valid procedure nor can it lead to any advancement of the theory of colloidal behavior. It appears reasonable to assume that if the kinetic considerations which underlie the deduction of Arrhenius' equations are both necessary and sufficient, the "constant" θ is truly constant. When, with a given set of conditions (as in our experiments at or near the isoelectric points for *Bact. coli*) the viscosity values calculated from the equation depart from the measured values it may be more reasonable to assume that the value of ϕ rather than the value of θ has changed. That there are good grounds for such a consideration has already been indicated in the remarks concerning Smoluchowski's work. We have also called attention to the finding that the bacteria agglutinate at or near the acidic and alkaline isoelectric points.

It is significant to recall that when bacteria (or other colloid-like particles)

agglutinate or "coagulate" the clumped aggregate and the dispersion medium occluded in it behave as a unit not only when there is streaming in the fluid due to mechanical forces, but even when there is opposite streaming of the aggregate particles and of the menstruum in an electrical field (cataphoresis). These considerations lead to the conclusion that the volume occupied by the dispersed phase is *apparently* increased by the volume of occluded water, although the *actual* volume occupied by the particles (bacteria) is unchanged. In Table V data are presented which show what the apparent relative volume of the dispersed phase (ϕ) would be near the two isoelectric points if the value of the constant in Arrhenius' equation be considered the same (6.0) at all pH values.

TABLE V. *The Values of ϕ (Calculated) When θ of Arrhenius' Equation is Equal to 6.0.*

Concentration (billions per cc.) (1)	Volume of Bacteria (ϕ) (2)	η Relative at pH = 3-4 (Table III) (3)	Volume of Disperse Phase at pH = 3-4 (ϕA) (4)	pH = 3-4 Ratio $\dfrac{\phi A}{\phi}$ (5)	η Relative at pH = 13 (Table IV) (6)	Volume of Disperse Phase at pH = 13 (ϕB) (7)	pH = 13 Ratio $\dfrac{\phi B}{\phi}$ (8)
5	0.00246 cc.	1.014	0.00101 cc.	.41	1.188	0.01247 cc.	5.07
10	.00491 cc.	1.078	.00544 cc.	1.11	1.169	.01130 cc.	2.30
17	.00835 cc.	1.152	.01026 cc.	1.23	1.575	.03288 cc.	3.94
50	.02460 cc.	1.506	.02964 cc.	1.20	4.185	.10360 cc.	4.21

It is apparent in the table that the ratio of the apparent volume of the dispersed phase near the acid isoelectric point to the volume calculated directly from the dimensions and the concentrations of *Bact. coli* $\left(\dfrac{\phi A}{\phi}\right)$ is about 1.2. (The ratio for the suspension containing 5 billions per cc. is less than 1. This is probably due to an experimental error.) It is also seen that near the alkaline isoelectric point the ratio $\dfrac{\phi B}{\phi}$ varies from 2.3 to 5.07, with an average value of 3.88.

We have conducted a series of measurements to determine the relative volumes occupied by the clumps of bacteria which are observable under the microscope in suspensions at pH = 3.6-3.8 and at pH = 13.0 and the actual volume occupied by the bacteria in the clumps. The gross volumes occupied by the clumps (bacteria plus occluded water) were calculated from average measurements of the outside dimensions of the clumps; and the volumes of the bacteria in the clumps from the average number of bacteria in each average clump and from the known average volume of each bacterium. In the acidulated solutions, the ratio $\dfrac{\text{volume of clump}}{\text{volume of bacteria}}$ is approximately 67; in the alkalinized solutions, the ratio is approximately 87. Hence, it follows that if even a small proportion of the bacteria in a suspension are in clumps the apparent volume of the dispersed phase is considerably increased. The apparent increase in the relative volume occupied by the bacteria we believe accounts for the departure of the experimental from the calculated viscosities.

Inasmuch as the agglutination of bacteria is, in a large measure, determined by the magnitude of the potential difference between them and their

menstruum it appears that the increases in viscosity of bacterial suspensions which follow upon their agglutinations are probably related to or, possibly, are even referable to the effect of pH upon the potential. If it be considered that the potential takes its origin primarily in ion equilibria of the type described by Donnan, it follows that the viscosity of bacterial suspensions—and perhaps of colloidal suspensions in general—are functions of the Donnan equilibria, in so far as these equilibria serve to affect the apparent volume of the dispersed phase. Further, it appears to be unnecessary, as Loeb has done, to postulate the production of submicroscopic particles to account for increases in viscosity caused by suitable alterations of hydrogen ion concentration. These effects of [H⁺] can be more simply—and, it appears to us, more reasonably—explained as consequences of the influences of [H⁺] (or other ions) in causing agglomeration of particles and consequent apparent increase in the volume occupied by the dispersed material.

In conclusion, we may say that the viscosity, like certain other properties, of bacterial suspensions appears to be determined by the colloidal nature of bacteria and can be treated as though the bacteria were inert material in the colloidal state. *Pari passu*, studies on viscosity indicate the length to which certain properties of bacteria can be analyzed along the lines of colloid chemistry.

IV. Reactions of Bacteria with Various Reagents

It has been supposed by many investigators that the reactions of bacteria with lethal or non-lethal reagents can be divided into two steps, the first being concerned with the absorption of the reagent by the bacterium and the second with the reactions between the reagent and the protoplasm; that the first is dependent upon the dynamics of colloidal behavior and that the second is probably very complex and is not resoluble by the dynamics of simple homogeneous or heterogeneous equilibria. If the bacteria be considered colloidal particles they may react with the reagent in one or more of four ways: (1) A new chemical compound may be formed between the test reagent and components of protoplasm; (2) there may be a distribution of reagent between menstruum and bacterium according to Henry's law; (3) there may be distribution according to the adsorption isotherm; (4) there may be distribution according to the Donnan equilibrium. Herzog and Betzel [22] reported that the reaction between yeast cells (which are closely related to bacteria) form a chemical compound with formaldehyde, whereas they take up chloroform or silver nitrate according to the adsorption isotherm. Reichel [23] concluded that phenol was distributed between bacteria and menstruum according to Henry's law. Norton and Hsu,[24] studying the disinfectant action of formic acid and its distribution between bacteria and water, reported close adherence to the adsorption isotherm. The disinfectant action was considered to be due to the adsorbed hydrogen ions. Morawitz [25] had also come to the conclusion that the action of certain poisons on microörganisms is proportional to the amount deposited on their surfaces.

The dynamics of a lethal reaction between bacteria and a disinfectant appear, under certain conditions, to follow those of mass action reactions.

[22] *Z. physiol. Chem.*, **67**, 309 (1910).
[23] *Biochem. Z.*, **22**, 149 (1909).
[24] *J. Inf. Dis.*, **18**, 180 (1916).
[25] *Koll. Chem. Bei.*, **1**, 301 (1910).
[26] *J. Bact.*, **11**, 1 (1926).

Thus, in a recent review of this problem, Falk and Winslow [26] concluded that the mortality of *Bact. coli* in solutions of NaCl and $CaCl_2$ and in distilled water of varying pH follows a generally logarithmic course and may be roughly described by the equation of an unimolecular reaction, as has been shown to be the case for other processes of disinfection. The time curve closely follows the relation:

$$0.434\, K_1 = \frac{1}{t} \log \frac{a}{a-b} \tag{4}$$

where

$$\begin{aligned} K_1 &= \text{the velocity constant} \\ a &= \text{the original number of viable bacteria} \\ a - x &= \text{the number after time } t \\ t &= \text{time} \end{aligned}$$

This relationship is not, however, a close or an exact one, more or less marked deviations from the logarithmic curve becoming demonstrable on close analysis of the data. With NaCl the rate of the reaction seems to increase at first. With both salts, and with acid and alkali, it decreases gradually as the disinfection progresses through its later stages. It was found that where disinfection does not follow a logarithmic course and is not to be described by the equation of an unimolecular reaction, the course may sometimes be described by the equation of a bi-, tri- or higher multimolecular reaction. Such an explanation renders unnecessary the assumption, which has been made by many investigators, of differences in resistance of the individuals in a bacterial population to the lethal agent to account for deviations from a logarithmic mortality curve. It is also interesting to note that when used in sufficiently low concentrations, NaCl and $CaCl_2$ may be not only without toxic properties for *Bact. coli*, but may even stimulate the organisms to increased growth and reproduction. For a series of concentration of $CaCl_2$ acting upon suspensions of *Bact. coli* a curve may be plotted which shows continuous gradations between intoxicating and stimulating effects. The characteristics of this curve may be approximately defined by the equation:

$$K_1 = 0.36 \log^2 C + 1.6 \log C - 1.5$$

where K_1 is the unimolecular velocity coefficient and C is the molar concentration of salt.

If the disinfection of a bacterial suspension by $CaCl_2$ or other lethal agent is treated as a reaction in a heterogeneous instead of a homogeneous system and if the course of the process is found in accord with the logarithmic law, the significance of these findings becomes radically different from that which has been commonly placed upon them. They may mean that in the changes which lead to the loss of viability by bacteria a number of reactions (physical or chemical or both) are involved and that the slowest (and determining) one may be of the nature of a diffusion reaction or of any one of many kinds of multi- or interphase reactions (*vide* Taylor [27]). The precise treatment of these conditions was not possible with the data available.

The extreme sensitivity of bacteria to the hydrogen and hydroxyl ions suggests the amphoteric nature of these organisms. The demonstration of a buffering action of bacteria towards these ions has been carried out by

[27] Taylor, H. S., "A treatise on physical chemistry," New York, Vol. II, 933, 1924.

Shaughnessy and Falk.[28] They conducted a series of electrometric titrations on suspensions of bacteria in water and in salt solutions. From comparisons of these curves with similar titration curves for the water or the salt solutions alone they calculated the buffer exerted by the bacteria. They found that in distilled water *Bact. coli* possesses distinct buffering capacities which are greatest in the most favorable zone for viability—pH 6 to 6.9—and which are significant in the range which is of physiological importance— pH 4 to 10. Above pH 10 and below pH 4 the buffer ratio approximates unity and further may be so significantly below unity as to suggest that there is actual liberation of, rather than reaction with, the hydrogen or hydroxyl ions which predominate in the surrounding medium. The existence of the second, alkaline isoelectric point is suggested because the buffer becomes insignificant several pH units away from the acid isoelectric point (which is below pH 1.0 for this strain) and likewise becomes insignificant at certain alkaline pH values. (The existence of alkaline isoelectric points was actually demonstrated later by cataphoresis experiments.[29] NaCl and CaCl₂ reduced the buffering capacities of the bacteria, the latter being more effective. The depressing effects of salts were particularly evident in neutral or alkaline solutions. This observation appears to be in harmony with deductions which may be drawn from studies on the electrophoretic potentials on bacteria. Thus, it has been clearly established that the bacterial cell in neutral solutions is electronegative to water. This is entirely in accord with the well-known fact that bacteria are especially reactive with electropositive ions and are the more reactive with them the more alkaline the solution up to certain limits. The differences in the observed behavior of sodium and calcium chlorides must be considered as due to the specific properties of the cations of these salts which dissociate to give a common anion. When the menstruum is rendered acid the electronegativity of the cell is reduced (and is abolished when the acidity attains the specific value which is termed the "isoelectric point"). Its reactivity with cations—it may be expected—will be diminished. Hence the reactions with sodium or calcium cations which are evidenced by depression of the buffer ratio of the bacteria should be, as they are, more marked in neutral or alkaline than in acid solutions.

In an entirely similar manner, Stearn and Stearn [30] have utilized the amphoteric, colloidal behavior of bacteria to account for the differences in the reactions of bacteria to acidic and basic dyes in solutions of various pH values.

[28] *J. Bact.,* **9**, 559 (1924).
[29] Winslow, Falk and Caulfield, *J. Gen. Physiol.,* **6**, 177 (1923); Winslow and Shaughnessy, *ibid.,* **6**, 697 (1924).
[30] *J. Bact.,* **8**, 567 (1923).

Colloids and the Growth of Microorganisms

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This essay does not pretend to give a detailed account of all that has been done on the subject under consideration. The object has been to outline the general problems involved, using such illustrative material as seems particularly applicable, and to suggest methods of approach to problems so far not investigated. By adopting such a plan the author hopes to call attention to a field of research with too few workers and one in which there is ample opportunity to be of service to science.

The most striking characteristic of microorganisms, the one which first attracted the attention of the scientifically curious, is their ability to reproduce. This process does not consist in the mere fragmentation of protoplasm, but it is accompanied by an actual accumulation of life stuff. The new individual is in all apparent ways identical with the cell from which it was derived.

The living cell might be regarded as an evolutionary energy transformer which maintains itself by a complex of harmonious chemical reactions taking place in the proper colloidal environment. Bechhold compares the living cell "to a city, in which the colloids are the houses and the crystalloids are the people who traverse the streets, disappearing into and emerging from the houses, or who are engaged in demolishing or erecting buildings. The colloids are the *stable* part of the organism; the crystalloids the *mobile* part, which penetrating everywhere may bring weal or woe."

However, the living cell is more than a heterogeneous system, or a series of reactions; life is an event, a coincidence, the meeting point of many curves, characterized by a harmony among a large number of factors. Life is a whorl of interlocking chemical reactions striving at an equilibrium, the attainment of which would mean death. When the peculiar life harmony "happened," there were three possibilities before it. There might be lack of materials or energy for the maintenance of the life stuff, in which case life would soon cease. There might be a perfect balance of intake, output, and of protoplasm, in which case there would be a constant quantity of life with attendant risk of destruction through accident. Or the very process of coordination or harmony in maintaining itself might organize other matter, and the sum of life would increase. This last case is obviously the most important one, although there may continually be instances of the first two.

If an increase in the amount of protoplasm were a necessity for the maintenance of life, this excess could be taken care of in one of two ways: The cell might become indefinitely large, or there might be a breaking up into further units, i.e., reproduction would result. There are obvious limitations to the possible size for the existence of a cell, such as maximum size for intake of nutrient and elimination of waste products, the surface tension

of the system, osmotic influences, electrical condition, stiffness of wall and so forth. When this maximum size is reached the cell must die or divide. Given increase of protoplasm and the possibility of reproduction, the fact of different characteristic size of cells is a detail. Thus, it is not unreasonable to suppose that reproduction is not the fundamental property of life, but that it only serves as a device for taking care of excess protoplasm formed of necessity in the maintenance of life.

When the microörganism is introduced into a medium, there normally follows a sequence of reproductive periods termed growth phases. The classification of these growth phases is rather arbitrary, but, according to Buchanan (1918), in general there are seven principal phases involved. These are the initial stationary phase, the lag phase, the logarithmic phase, the phase of negative growth acceleration, the stationary phase, the phase of accelerated death, and the logarithmic death phase.

Changes in colloidal condition within or without the cell may affect any of the growth phases. In order to simplify the discussion only alterations in the logarithmic growth and in total crops will be considered, since these cases will not only illustrate general principles, but also because maximum rates of reproduction and total yields are of fundamental interest in the industrial use of microorganisms. Illustrations will be drawn largely from studies on yeast; first, because of the relatively large amount of research on this organism, and secondly, because the author has been engaged for some time with studies on yeast and feels more able to discuss this material.

During the logarithmic phase of growth the number of cells is increasing a certain percentage of itself each instant. It is simply a case of compound interest compounded at each instant. If x represents the number of cells, and t expresses the time, the rate of growth is expressed by the differential, $\dfrac{dx}{dt} = kx$, from which it follows that $lnx = kt + C$. In any case, the value of C is simply the value of lnx when $t = o$, or it is the logarithm of the original seeding. When lnx is plotted against time, there results a straight line with slope k, the value of whose intercept on the ordinate is C or the logarithm of the initial seeding. If $n =$ period of reproduction or generation time, then $n = \dfrac{ln2}{k}$. In the usual case of the plotting of logarithm to the base ten, it follows that $n = \dfrac{0.3010}{k_{10}}$. The above relationships have proved a most useful tool in microbiological studies, and have been mentioned here in the hope that their utility may be more widely recognized. The use of such formulations with reference to yeast may be illustrated by the work of Slator, Fulmer, Clark, and others.

The logarithmic phase is followed by a period of decreasing rate of growth until the maximum crop for the given conditions has been attained. The causes of this change in rate with reference to yeast have been studied, particularly by Slator, Fulmer, and Clark. A consideration of these findings will be of general applicability. The decrease in the rate of growth is caused by the action of accumulated volatile products of fermentation, and the change is coincident with the formation of a definite concentration of alcohol under given conditions.

Moreover, it was found that the cell underwent several fundamental

changes at the critical concentration of alcohol. It had been known for a long time that an old cell is much more resistant to a poison than a cell at the maximum rate of growth, but it is interesting to note that this change is not cumulative but sudden. That the resistance to phenol increases several hundred per cent. in a range of a few tenths of a per cent of alcohol developed in the medium, may be illustrated by data obtained by the author (1921) on the resistance of yeast cells to phenol as evidenced by the time required for 0.65 per cent. phenol (0.65 gram of phenol per 100 cc.) to cause 100 per cent of the cells to become permeable to methylene blue. The progress of the fermentation was measured in terms of grams of carbon dioxide evolved per 100 cc. of medium. The following table gives a typical series:

Grams CO_2 evolved	0.02	0.17	2.71	3.37	4.13	4.70	6.06	6.34
Staining time (minutes)	25	23	24	25	52	79	120	121

The failure to recognize the above discontinuous function of the metabolic product has led to confusing results in the comparison of the resistance of various strains of microörganisms to poisons. The slowing down of reproduction and other vital capacities of the cell due to accumulated metabolic products is universal, and in some cases acts to such a degree as to result in the death of the cell. In other instances, as in yeast, the harmful effect is minimized by a thickened wall and by other changes in the cell, the activity of the organism remaining at a minimum until placed in a new medium with a low concentration of the toxic products.

Changes in colloidal environment as they effect the logarithmic or maximum rate of reproduction and total crops may be combined in nine possible cases as follows:

Logarithmic Rate of Reproduction:	Total Crop
Increase	No effect
Increase	Decrease
Increase	Increase
Decrease	No effect
Decrease	Decrease
Decrease	Increase
No effect	No effect
No effect	Decrease
No effect	Increase

In most researches on the subject of the influence of various factors upon the growth of microörganisms, no distinction has been made on the basis of the particular growth phases so affected. The term growth acceleration in many cases refers to the increase in crops estimated at an arbitrary point. A consideration of the nine possibilities tabulated above shows the advisability in all such studies of indicating the particular growth phase involved. In many instances of the industrial use of microörganisms, because of the greater relative time required after the logarithmic production for the formation of a given amount of product, the total crop is not the goal, the important phase for study being the logarithmic phase with the increase or even the decrease in total crop of no importance. In other processes, where time is not an important element, the increase of total crop is fundamental even if the logarithmic phase be decreased. In view of the above, it seems advisable to outline methods by which the two growth phases may be altered and to

point out, where possible, instances of their application. In order to simplify the discussion, it will be assumed that the limiting factor is not food supply. It will soon be apparent that the important factor in any case involves colloidal phenomena.

FACTORS INFLUENCING THE LOGARITHMIC GROWTH PHASE

The fact that the rate of growth of a microorganism is, within limits, affected by *osmotic pressure* is of common knowledge and need not be discussed in detail. Undoubtedly this factor is in many cases a necessary concomitant of other variables, and probably has been overemphasized. However, it is certain that outside of ranges which differ with the various organisms, changes in osmotic pressure lead to decreased growth rates due to changes in concentration of cell contents, which affect the optimum colloidal condition in the cell.

The influence of the *reaction of the medium,* pH, is being largely emphasized in the recent literature. As in the case of osmotic pressure, the effects of hydrion, *per se,* must be differentiated from effects due to other factors necessarily accompanying hydrion changes. It must be remembered that any alteration in hydrogen ion concentration must be accompanied by change in the concentrations of other ions, as well as by variation in osmotic pressure and the like. However, that there is an optimum range of hydrogen ion concentration for the reproduction of microorganisms cannot be doubted. The effect of hydrion is probably felt through its influence upon the colloidal condition of the cell.

The surface tension (air-medium interface) may be an important factor in the growth of a microorganism. Organisms growing in a surface film will find an environment considerably different from that in the bulk of the liquid. Surface tension may likewise be significant in determining the type of growth in liquid culture. Larson (1921) reports that organisms may grow on a film on the surface of a broth with relatively high surface tension, but may not form a film if the surface tension is sufficiently depressed by appropriate solutes. The bacteriological significance of surface tension has been treated at length by Buchanan and Fulmer (1928).

The nature and concentrations of substances in the film may modify the rate of gaseous diffusion and of evaporation. For example, the oxygen concentration in a medium will necessarily depend upon the rate at which the gas is removed due to cell metabolism, and the rate at which it will diffuse into the medium from the air. If the film has in it a higher concentration of solutes than the bulk of the liquid, it may act as a membrane modifying the rate of diffusion of the oxygen. It is conceivable that such a situation might constitute a limiting factor for growth. The effect of surface tension upon rate of diffusion of gases through the surface has apparently not been adequately studied.

That rate of evaporation from a surface is altered by surface tension, is shown by data obtained by du Noüy (1926) in his studies on the effect of blood serum on the rate of evaporation. He found that a concentration of 1 : 10,000 of serum showed a maximum depression of evaporation. He explains the occurrence of this maximum effect by stating that "at the higher concentration crannies may exist, due to the disorderly piling up of the molecules, and at the weaker concentrations the surface of the liquid will not be entirely covered."

The second interface of interest is that of the microorganism and the medium. It cannot be assumed that a material which affects the surface tension (medium/air interface) will affect the interfacial tension (microorganism/medium interface) in the same way, an assumption that has at times been made. For example, Lewis (1909) found that some salts which increased surface tension, decreased the interfacial tension between a hydrocarbon and water. Bayliss (1920) makes the rather sweeping statement that "any substance dissolved in water lowers the surface tension at the interface between the solution and a solid or immiscible liquid." Freundlich (1924) cites several cases in which a knowledge of surface tension effects is untrustworthy in predicting the types of adsorption at liquid or solid interfaces. Moreover the effects of materials upon surface tension may not be additive. Berczeller (1917) found that salts which raised the surface tension of water have the opposite effect when added to solutions of gelatin and other proteins. Furthermore, the addition of salts to dilute phenol solution produces a lowering in surface tension even greater than that of a protein solution. Evidently the effect of added materials upon surface tensions cannot safely be used to predict changes in interfacial tensions.

Substances which lower the interfacial tension between the microörganism and medium will tend to concentrate (be positively adsorbed) at the interface; the opposite tendency exists for materials raising the interfacial tension. This applies of course to the metabolic products as well as to the original constituents of the medium. Rideal (1922) states that "if during the growth of the organism substances are formed which tend to lower the interfacial surface tension in a marked manner, then, in accordance with the general theorem of mobile equilibrium of LeChatelier if the chemical process involves the possible formation of such substances, there will be a tendency for their production, and these substances will be adsorbed on the surface of the bacterium and affect the growth rate."

It follows at once that the addition to the medium of a material which is more strongly adsorbed than the metabolic product, will tend to replace it and thus nullify its action on growth. In cases where the decreased interfacial tension increases rate of reproduction, it would be interesting to know whether the cell size may be smaller without corresponding increase in protoplasmic material. If this be true, it would lend credence to the view that the interfacial tension is one determinant of cell size. It will be pointed later that in some instances, results on growth attributed to interfacial tension alone, may be due in part at least to other accompanying factors.

That there is a relation between the rate of growth and the *permeability of the plasma membrane*, is indicated by the work of Boas (1921-1922) on the effect of saponins on fermentation by yeast. He found that certain saponins in low concentrations stimulate the fermentation rate, while higher concentrations are inhibitory. The increase in rate of fermentation with increased permeability is attributed to the possible increase in the rate of diffusion of sugar and of metabolic products through the membrane.

There is another way in which changes in permeability may alter growth rate. It has been stated that life maintains itself by a series of interdependent chemical reactions taking place in the proper colloidal environment. Each reaction has its own velocity coefficient, the amount of product formed depending upon the rate of delivery from a previous process. The leakage of a product due to increase permeability will cause the acceleration in all pre-

ceding processes in order that the proper balance may be maintained. This in turn might lead to so pronounced an acceleration of activities as to markedly increase the rate of reproduction. If the permeability be too greatly increased, the balance is so upset as to lead to death, i.e., a small amount of an added material may accelerate growth, while a larger concentration proves toxic. In cases where the permeability of the cell is decreased, there might result a lessened rate of growth due to the delivery of an excess of material at a given point in the complex of reactions. A study of growth accelerators as they affect cell permeability might lead to the classification of a considerable number under this head.

In the course of studies on the relation of the composition of the medium to the growth of yeast Fulmer, Nelson, and Sherwood (1921) found an apparent relationship between rates of reproduction of the microorganism and the *extent of hydration of the cell colloids*. The evidence is presented by the behavior of yeast in the presence of varying concentrations of ammonium salts. It was found that there is for each ammonium salt tested an optimum concentration for the growth of yeast at a given temperature, and that this concentration in each case is at the same normality, the effect being due to the ammonium ion. Moreover, the concentration required for maximum reproduction rate is greater with increasing temperature. It is of interest to note also that the growth of yeast in wort is improved by an optimum concentration of ammonium chloride, and that this concentration is affected by temperature similar to that shown in a synthetic medium (1924).

A study of the effect of varying concentrations of ammonium chloride on the swelling of wheat gluten disclosed the fact that at all temperatures tested the concentration of ammonium salt for the maximum reproduction of yeast, was identical with that concentration in which the gluten was the least swollen. The figures in Table I will be illustrative of typical data.

TABLE I. *The Effect of Ammonium Chloride on the Growth of Yeast and upon the Swelling of Wheat Gluten.*

Normal-ity	21° C.		30° C.		35° C.		40° C.	
	Yeast Millions of Cells per cc.	Gluten Loss in Wt. per 1000 g.	Yeast Millions of Cells per cc.	Gluten Loss in Wt. per 1000 g.	Yeast Millions of Cells per cc.	Gluten Loss in Wt. per 1000 g.	Yeast Millions of Cells per cc.	Gluten Loss in Wt. per 1000 g.
	..	37	..	28	..	24	..	36
0.0018	1.5	37	2.5	..	6.2	51	4.0	60
0.0177	1.7	56	3.0	62	..	..	4.5	..
0.0236	2.3	76	4.0	76	7.0	77	..	73
0.0266	2.5	..	4.5	..·	..	..	..	..
0.0295	4.5*	84*	5.5	84·	15.0	79	12.0	75
0.0325	2.25	..	7.5	..	16.2	..	..	..
0.0353	1.7	60	9.5*	90*	17.0	88	19.0	83
0.0382	..	55	5.0	..	22.0*	109*	21.5	92
0.0412	..	50	4.7	60	17.0	70	27.5*	99*
0.0442	..	..	..	..	..	..	19.0	72
0.0472	1.5	33	2.7	20	14.2	61.2	15.5	..
0.0531	..	..	..	..	12.5	..	12.5	..
0.0590	..	..	..	..	..	21	..	55

* Indicates maximum effect.

In the case discussed the microorganism exhibited the greatest rate of growth in a medium favorable for the minimum amount of hydration of protein. It is evident, then, that the ammonium ion plays an important rôle with reference to the rate of reproduction, and that the effect is connected with its influence upon the hydration of cell colloids.

Increase in *temperature* up to a certain point increases the rate of growth of microorganisms. From the work discussed it is apparent that increasing the temperature of the medium with a constant concentration of ammonium salt affects the growth of yeast and the swelling of a protein in the same way as decreasing the concentration of the salt at constant temperature. The salt acts as a temperature "buffer," permitting rapid growths of yeast at temperatures heretofore considered to be well above "normal." It is evident, then, that the temperature coefficient of the growths of microorganisms is misleading unless the above facts are taken into consideration. It should also be noted that data obtained on the effect of various reagents upon the swelling of colloids at one temperature cannot be safely made the basis for conclusions regarding other temperatures. As in the case of the yeast, the effect of temperature alone cannot be determined in a medium of constant composition. These results have been summarized by the author (1925) and by Sherwood, Nelson and Fulmer (1926).

In summation it may be stated that the logarithmic rate of reproduction may be influenced by changes in osmotic pressure, in the reaction of the medium, in surface tension, in permeability, in temperature, and in the state of hydration of cell colloids.

Factors Influencing Total Crops

It was stated previously that due to the influence of accumulated products of metabolism, the logarithmic rate was followed by a decreasing rate of reproduction until the total crop had been attained in the given medium. Any factor which will delay this effect will increase the crop and any methods of increasing the toxicity of the product will decrease the yield.

Aeration has been mentioned previously as a means for removing alcohol from a fermenting mixture and thus increasing yields. This method would apply to any volatile product. *Dialysis* or *electroendosmose* may serve a similar purpose. The manufacture of vinegar by the "quick vinegar" process keeps the concentration of acetic acid low enough to permit maximum action by the bacteria involved. Similar methods of continuous process might be more generally applied than at present to the industrial production of microorganisms and of their products.

The toxic material may be *precipitated* by some constituent of the medium. For example, the presence of calcium salts will, by forming the relatively insoluble salts, enable organisms producing oxalic acid or citric acid to form larger quantities of these materials, and hence reach larger total crops. The application of this factor would, in many cases, seem to offer promise of utility in large quantity production. In cases where the precipitating agent would be injurious if added at once, the material might be made available in several doses.

Where the toxic effect is due to changes in pH, the effect may be *neutralized* by the addition to the medium of proper neutralizing agents or buffers, and the crop increased by this method.

There may be formed *non-toxic, non-diffusible materials* such as gluco-

Cortical Reactions and Attendant Physico-Chemical Changes in Ova Following Insemination

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The first visible changes in the animal egg following insemination are in the cortex*   These cortical changes differ in eggs of different species, the difference being regarded as an expression of the physico-chemical make-up of the eggs themselves.   Fundamentally, however, all ova respond to insemination with some kind of cortical change; this response (which I have studied in several types of eggs under normal and varying experimental conditions) is important for the analysis of what I have called the fertilization reaction, the initial event in the development of which the visible cortical changes are sequelae.

Description of Cortical Changes in Some Types of Ova

The eggs of sea urchins have been the classic object for the study of cortical changes—for example, the "formation of the fertilization membrane," as it is often erroneously called.   Fol in his great work on fertilization described these changes.   Since Fol, any number of workers have studied these changes, especially in eggs used extensively in physiological experiments.   We may, therefore, describe the cortical changes that take place in the egg of the common American sea urchin, Arbacia.

The unfertilized egg of Arbacia is enclosed in a soft transparent jelly hull about one fourth the diameter of the egg.   Beneath this jelly hull, closely adherent to the surface of the egg, is a delicate (vitelline) membrane.   The cortex lies under the membrane.   Evidence can be adduced to show that this cortex is different physiologically from the interior of the egg.

About thirty seconds after the eggs and sperm are mixed, beginning at the point at which the sperm attached itself to the egg surface, the membrane begins to lift and progressively separates from the surface of the egg.   There thus arises between the egg surface and the membrane the space called the perivitelline space.   This increases in width so that in about one hundred and twenty seconds after mixing eggs and sperm it is equidistant from the egg at all points.

This progressive lifting of the membrane, first described by Fol, has been generally disregarded by workers, due largely to the influence of those who without really any good evidence have argued that the membrane is a new

* For a more detailed account of these changes under normal and experimental conditions as well as for the best account of the whole question of fertilization the reader should consult the excellent little book, Problems of Fertilization," by Frank R. Lillie, University of Chicago Press, 1919.   See also, "Fertilization," by Frank R. Lillie and F. E. Just, in "General Cytology" (edited by Cowdry), University of Chicago Press, 1924.

formation arising as a result of insemination. Thus it has been called the "fertilization membrane" even when its separation from the egg has been provoked by any one of a number of agents, both physical and chemical. I
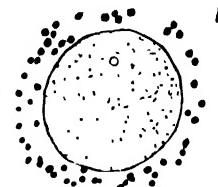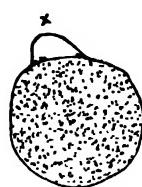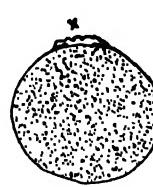
FIG. 1. FIG 2. FIG. 3.

FIG. 1.— Freshly shed egg. Its pigmented jelly is as yet unswollen by the sea water.
FIG. 2.—An egg ten seconds after the disappearance of the sperm head within the egg.
FIG. 3.—Same as Fig. 2.
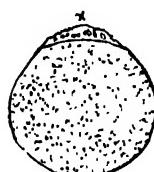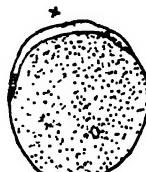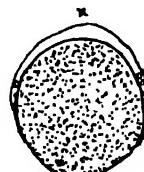
FIG. 4. FIG. 5. FIG. 6.

FIG. 4.—Four seconds after the membrane began lifting. Free vesicles beneath the membrane.
FIG. 5.—About two seconds later than Fig 4.
FIG. 6.—Two to three seconds later than Fig. 5. Note vesicles forming.
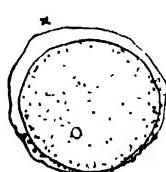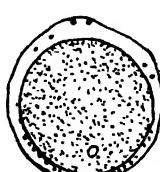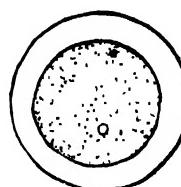
FIG. 7. FIG. 8. FIG. 9.

FIG. 7.—About two seconds later than Fig. 6. Membrane incomplete at pole opposite entrance-point of the sperm.
FIG. 8.—Twelve seconds after beginning of membrane lifting. The membrane is fully formed but still wrinkled, especially at point at which it lifted last.
FIG. 9.—Two minutes after insemination.

have, however, seen the membrane separate in a wave-like progression from the egg of Arbacia countless numbers of times, though it is not the most favorable form for this study. In the egg of Echinarachnius, the common sand dollar, the separation of the membrane is readily followed.[1]

[1] Just, E. E., *Biol. Bull.*, **36**, 1 (1919).

If eggs of Echinarachnius be inseminated with thin sperm suspension, they throw off membranes that are fully formed and equidistant from the egg surface in about two minutes. This follows sperm penetration. The sperm head having completely disappeared within the egg, a blister appears on the egg surface at the point of sperm entry. This blister contains drops that move toward the membrane and go into solution. This process is gradually continued throughout the whole cortex, the membrane arising in a wave that sweeps over the surface. By breakdown of cortical material beginning at the site of sperm entry, the membrane is separated from the surface. The accompanying figures (Figs. 1 to 9) illustrate the process as seen under the low power of the microscope.

After the membrane is fully off the egg, there forms over its surface a layer known as the ectoplasmic or hyaline-plasma layer. This is the semipermeable membrane of the cell. The vitelline membrane once off, no longer has any functions of the living substance.

The cortical changes in the egg of Nereis are worthy of note. Before insemination this egg possesses a thick cortex of alveolar structure. When eggs and sperm are mixed, sperm attachment rapidly ensues, bringing about the escape of material from the cortex which rapidly sets in the sea-water as a transparent jelly. The cortical alveoli thus break down, leaving only strands that cross the space between the membrane and the egg. Now comes a period of amoeboid changes in the egg: it shrinks, becomes markedly irregular in outline, its contents become darker, and the perivitelline space much reduced. When this period passes over the egg assumes a clearer and more rounded appearance; the perivitelline space widens again. There is a delicate plasma membrane beneath the vitelline membrane.

There are here, therefore, cortical changes of a striking character unlike those observed in the eggs of Arbacia and of Echinarachnius. In all three the essential response to insemination is the same; namely, an alteration of the cortex. This phenomenon is important in the physiology of fertilization as shown by studies on cross fertilization and experimental parthenogenesis; cortical changes play a leading rôle in the initiation of development in animal ova no matter what the agent of initiation may be—sperm of the species, foreign sperm, or agents of experimental parthenogenesis.

Altered Physical State of Ova during Cortical Changes

The morphological changes in the cortex of the ova just cited may be correlated with an altered physical state which has been investigated chiefly by Just.[2] He finds that the egg of Echinarachnius is about twelve times more susceptible to exposure to dilute sea-water during the period of membrane separation, than the inseminated egg before or after membrane separation, or than the uninseminated egg. The egg is never again so susceptible, though during the cleavage cycles it exhibits rise and fall in susceptibility which may be correlated with the mitotic phenomena.[3]

We may consider in detail this altered physical state first as seen in the egg of Echinarachnius.

If a drop of uninseminated eggs of Echinarachnius in sea-water mounted under the low power of the microscope be flooded with tap or distilled water,

- Just, E. E., *Am. J. Physiol.*, 61, 516 (1922).
- Just, *Ibid.*, 61, 505 (1922); Phys. Zool., 1, 26 (1928).

they take up water, swell, and finally break down in two or more minutes after flooding. Thus the time to disintegration in tap water for lots of eggs from ten females was as follows:

Number of female........	1	2	3	4	5	6	7	8	9	10
Time in seconds to disintegration of the eggs while in tap water......	270	243	60	113	150	148	256	247	155	240

The rate of disintegration in eggs from these same females exposed to the action of tap water five to ten seconds after insemination was about the same. With the beginning of membrane separation, twenty to thirty seconds after insemination, it is quite otherwise.

The inseminated egg during the process of membrane separation. Eggs from these same ten females were inseminated and exposed to tap water during the period of membrane separation. The following figures show that the eggs are highly susceptible at this time:

Number of female........	1	2	3	4	5	6	7	8	9	10
Time in seconds to disintegration of the eggs while in tap water	14	17	19	20	9	11	18	16	7	6

The inseminated egg two minutes after insemination. Eggs from the same females exposed to the action of tap water two minutes after insemination, i.e., after the membranes are completely off the egg, withstood the action of tap water better than the uninseminated eggs. With the full separation of the membrane the egg shows highly increased resistance to cytolyic action.

Once it was well established with tap water that the susceptible period falls in exactly with the period of membrane separation, attention was directed to the susceptibility of the egg to less dilute sea water at different stages of the process of membrane separation. This seemed important because with the 100 per cent tap water it appeared that *when the egg cytolyzed, the break always came from that part of the cortex from which the membrane was lifting at the time of exposure.* The cytolysis with tap water was, however, far too rapid to be sure of this. The less dilute sea water proved that this interpretation was correct. *When eggs are exposed to dilute sea water during the period of membrane separation they cytolyze by an outflow of cytoplasm at the points from which the membrane is lifting at the moment of exposure.* But what is more important is that like the process of membrane separation, the susceptibility travels at the same rate as the membrane lifting wave. As the cortex goes into solution, thus lifting the membrane—a process easily followed under the microscope—droplets of ectoplasm move across the perivitelline space before they completely disappear. Any point on the egg surface where this dissolution is taking place becomes the point of susceptibility at the instant of the dissolution.

In the next place, one must note the most remarkable characteristic of this period of susceptibility during membrane lifting. The susceptibility of the cortex is rapidly reversible. I have made observations on thousands of eggs and have yet to see an egg exposed during membrane separation break in those regions from which the membrane has not lifted. This is especially well brought out by exposing eggs during the early stages of membrane separation. Any part of the egg from which the membrane is not lifted is resistant

to the dilute sea water. And it is equally true that any part of the egg from which the membrane has lifted is resistant. This is strikingly shown by exposing eggs just at the moment that the membrane is being lifted from the last point on the egg surface. In this case the breakdown is only at this point; the zones from which the membrane is already off are resistant. When the membrane is fully off, the egg leaves the period of susceptibility. We may say, therefore, that a wave of resistance to dilute sea-water follows in the wake of the wave of susceptibility. There is an exceedingly rapid restitution process in the cortex following a momentary loss of resistance through the normal cortical breakdown that pushes off the vitelline membrane.

The uninseminated egg when subjected to the action of tap water becomes granular and disintegrates. At one point in the cortex just under the membrane the cytoplasm forms bubbles. These gradually form throughout the egg. The membrane now stands out as clearly as the so-called "fertilization membrane." After the cortex goes into solution in this way the interior of the egg is destroyed. The cytoplasm resembles crystals of salt through which water is seeping, so granular is its appearance. The effect of the tap water in lifting the membrane is often lost sight of because the swelling of the cytoplasm is so closely connected with the breakdown of the cortex that the water-logged cytoplasm fills out the distended membrane. Disintegration now takes place *within* the membrane. This is the usual procedure. Occasionally, however, the egg ruptures at one point and cytoplasm pours out at this point from the egg. The two striking characteristics of the behavior of the uninseminated egg in response to the treatment with tap water are the granular appearance of the cytoplasm and the general uniform disintegration of the egg. It is as though the cytoplasm is washed away.

With the inseminated egg during the susceptible period the case is quite different. The inseminated egg treated with tap water ruptures at one point and a portion flows out, which may form a ball after it flows out and so maintain itself in sea-water for some time. The cytolyzed plasma tends to cohere. This outflow usually does not pass beyond the membrane, which remains intact. Or, if the outflow forces itself through the membrane, the rest of the membrane remains intact and spherical. In other words, the physical properties of the inseminated and the uninseminated egg are quite different as shown by the difference in reaction to hypotonic sea-water. This change is apparent the *moment the membrane begins to lift* and can be very clearly revealed by the study of the behavior of the egg in hypotonic sea-water of graded strength.

If we attempt to come to some conclusion as to the meaning of this susceptibility we must take into account the following facts. First, the very rapid recovery of the cortex after membrane lifting; second, the resistance of the cortex from which the membrane is not yet lifted; third, the apparent failure of the cortex at the site of sperm to dominate the rest of the cortex in any way, either in greater resistance or susceptibility, or in persistence of the effects of the hypotonic sea-water. In brief, we must keep in mind that this susceptibility is clearly localized; or, what is more correct, that the break in the egg which is the expression of decreased resistance to the hypotonic sea-water is clearly at the site of membrane lifting. Now this does not mean that this is the point at which the water enters the egg. Rather, it means that the point at which he membrane is lifting is the point at which the cortex is weakest. We are not dealing with an imperforate membrane but rather *with the cortex* that is not continuous; it shows a break in the zone of mem-

brane lifting. The normal process of the cortical secretion is doubtless bound up with water movement. The cortex is washed away. The material goes into solution. Now, this process is one of rapid recovery not only in the cortex itself but toward the interior of the egg, for the interior of the egg never becomes involved. In the normal process just enough water is present to carry out the normal process, but when the egg is placed in dilute sea-water, the picture is different. The recovery in the zone of membrane formation cannot take place, and the cortex breaks. Moreover, with the cortex gone, the endoplasm is without the cortical protection and it too becomes involved so that complete cytolysis is the result. We are here dealing, then, with a sensitivity due to actual progressive dissolution of colloids in the cortex of the egg.

To the reader it is at once apparent that the process of membrane separation exhibited by the *Echinarachnius* egg closely resembles that of transmission in various tissues. The way in which the cortex responds to hypotonic sea-water may be additional evidence of this similarity. Certainly, the response of the cortex during membrane separation to the hypotonic sea-water reminds us of the action current in a stimulated nerve. The high susceptibility of the cortex in the zone of membrane separation and the relatively resistant zones from which the membrane has not separated suggest the electronegative condition of a nerve fiber set up wherever the excitation may be at a given instant as it sweeps along the fiber. It may well be that this is more than a superficial resemblance and that the study of this cortical response would warrant further investigation from this point of view.

The case of the egg of Arbacia is very interesting. Differences in resistaance to dilute sea water shown by the uninseminated egg and by the inseminated egg at the time of membrane separation are not clear cut. That the period of membrane separation is a critical one, however, is shown by a study of the swimming larvae reared from eggs treated with dilute sea water before, during, and after membrane separation. Inseminated eggs exposed to tap or distilled water before or after membrane separation and then returned to normal sea water give rise to normal larvae. But eggs exposed to the tap or distilled water during the period of membrane separation on return to normal sea water show marked abnormalities in the late stages of gastrulation. There is here, therefore, definite evidence of a differential susceptibility which appears in clear cut fashion; dilute sea water exerts a decidedly deleterious action during the process of membrane separation and the egg is thereby so profoundly altered that the normal processes of gastrulation are disturbed.

Without going into details, I may say that the uninseminated eggs of Neresis limbata withstand treatment with tap or distilled water for three or four minutes before disintegration. Beginning twenty-five minutes after insemination the eggs are even more resistant than this. During the twenty-five minute period immediately following the mixing of eggs and sperm the eggs show a very low resistance—disintegrating in from ten to sixty seconds after exposure to tap or distilled water. The cortical changes in this egg described above, the reader will recall, run over a period of twenty-five minutes. Thus, the period of susceptibility to hypotony exactly parallels the period of cortical breakdown with release of the jelly-forming material, the amoeboid changes, and the darkening of the egg.[4] When the egg rounds up and clears, the period of lowered resistance to dilute sea water passes off.

⁴ Just, E. E., *Anat. Rec.*, **20**, 225 (1921); *Phys. Zool.*, **1**, 122 (1928).

These experimental findings on three forms—and I have used eggs of other forms as well—though they show variations, nevertheless point to one conclusion: the period of cortical changes following insemination is one of profound physical alteration. Chemical changes take place at this time also.

OXYGEN CONSUMPTION

Oxygen consumption of ova at various stages of development following insemination has been investigated by several workers—Warburg,[5] Loeb and Wasteneys,[6] and Meyerhof.[7] More recently Shearer [8] has reported his findings on oxygen consumption in the eggs of sea urchins during the first minute after mixing eggs and sperm.

Says Shearer: "On addition of the sperm to the eggs there is an immediate consumption of oxygen. In the course of the first minute the uptake of oxygen is many times that of the same eggs one minute before the addition of the sperm, and more is usually taken up in the first minute than is taken up in the second and third minutes after the addition of the sperm taken together." Shearer thinks that this "great initial inrush of oxygen into the egg and a corresponding output of CO_2 within the first minute after the addition of the sperm" make it clear "that the spermatozoon sets up an instantaneous oxidation process in the egg, which is unparalleled in the reactions of the animal cell for its sudden character."

Assuming that Shearer's results are correct, we may conclude that the period of the great initial inrush of oxygen coincides with the period of cortical changes described above at which time the egg is so susceptible to the action of dilute sea-water. During this period the colloids in the egg cortex go into solution, material is destroyed. It is this destruction at the surface of the egg that is accompanied by the great rise in oxygen consumption.

But according to Shearer it takes sperm at least 10 to 15 minutes to enter the eggs. This is amazing, if true, and unlike the penetration of sperm into any sea urchin egg that I know. To be sure sperm remain external to the egg for about 40 minutes in the case of Neresis and about 20 minutes in the case of Platynereis. These are unusual cases; in most eggs sperm enter shortly after contact with the eggs—penetration is a matter of seconds. Shearer is doubtless in error here. This, however, would not affect my interpretation of his results if his error is one of observation; if sperm do actually remain outside for the time that he gives, then his eggs were abnormal.

HEAT PRODUCTION

Shearer [9] has, like Meyerhof [10] previously, investigated heat production in inseminated eggs of sea urchins. Recently, Rogers and Cole,[11] using methods of higher precision, have reported most important findings on the heat production by the eggs of the sea urchin, Arbacia. Their results on the heat production immediately following insemination are of most interest to us. These workers find that the rate of heat production at the instant of insemination is ten to twelve times that of the uninseminated egg. Thereafter the rate of heat production falls constantly for twenty minutes to 65 per cent of the value

[5] Warburg, O., *Z. Physiol. Chem.*, **57**, 1 (1908).
[6] Loeb and Wasteneys, *Biochem. Z.*, **37**, 410 (1911).
[7] Meyerhof, O., *Biochem. Z.*, **33**, 291 (1911).
[8] Shearer, C., *Proc. Roy. Soc.* (London), Ser. B, **93**, 213 (1922).
[9] Shearer, C., *ibid.*, **93**, 410 (1922).
[10] Meyerhof, O., *Biochem. Z.*, **35**, 246 (1911).
[11] Rogers and Cole, *Biol. Bull.*, **49**, 338 (1925).

at insemination, remaining constant for the next thirty minutes (to first cleavage) to drop again by more than 10 per cent, remaining constant as far as the eight cell stage—as far as Rogers and Cole carried their observations.

Figure 10 (from Rogers and Cole) gives the approximate *rate* of heat production. This is the average of seven runs. This figure would certainly bear the interpretation that the maximum heat output falls within a minute after the mixing of the eggs and sperm. In other words, "the greatest period of heat production occurs immediately upon fertilization" (Rogers and Cole, p. 350). This undoubtedly means that this period falls in with the period

Fig. 10.—Rate of heat production, *Arbacia* eggs Abscissæ, time in minutes after fertilization Ordinates, rate of heat production in calories per hour per million eggs (After Rogers and Cole)

of cortical changes with which we are concerned. Say Rogers and Cole: "The fact that the greatest heat production by the egg comes immediately after fertilization seems to us to make it plausible to say that the entrance of the spermatozoon induces a cortical oxidation process, and that this process results in the elevation of the fertilization membrane." In other words, what Rogers and Cole have measured is *the heat produced during the disintegration of the cortical colloids as it sweeps in a wave beginning at the site of sperm entry over the surface of the egg, thus separating the vitelline membrane.* Membrane separation is thus explosive in character.

Other physico-chemical changes—in permeability, viscosity, electrical conductivity, etc. - follow insemination; but these undoubtedly, in the eggs used, are subsequent to the cortical changes (membrane separation in the sea urchin egg, for example). Moreover, such changes are rhythmic, paralleling the cycles of cell division. The changes that I have discussed here are the result of the attachment of the sperm to the egg whereby a reaction takes place between the sperm head and a cortically located substance, fertilizin.[12] This reaction is practically instantaneous and irreversible, finally expressing itself in an explosion that sweeps over the egg, thus breaking down the cortex.

[12] Lillie, F. R., *J. Ex. Zool.*, 16, 523 (1914).

The Arrangement and Action of the Colloids of the Plant Cell

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The visible structures of the cell include a mass of hydrogels, which are reversible, inclusions not liquefiable in water without modification of their composition, and an outer envelope or wall. The hydration phenomena of all of this material must be taken into account in any consideration of growth and permeability. Research in metabolism has been directed chiefly to the proteins and their multifold derivatives. Information concerning the lipins or phosphatides, and pentosans or mucilages, is in a much less advanced state. Substances of these last two groups have been found to be much more abundant in plant tissues than in animals. The soaps are also to be considered as a minor element, which may not play a very important part in the phenomena of hydration, or in transformations of energy.

The general character of the living matter may be taken to be derived from the proportions of its constituents. Lepeschkin has recently repeated the analyses of Reinke made in 1881-83, and finds that the naked masses of the slime mould (*Fuligo varians*) have on the average of 82.6 per cent water. The material in solution, amounting to 40.7 per cent of the dry weight, is made up of monosaccharides 14.2 per cent, albumin 2.2 per cent, and amino-acids, etc., 24.3 per cent. The ground-mass of protoplasm which does not dissolve, and which makes 59.3 per cent of the weight of this organism, comprises nucleo-proteins 32.3 per cent, free nucleic acid 2.5 per cent, globulin 0.5 per cent, lipoproteins 4.8 per cent, neutral fats 6.8 per cent, phytosterin 3.2 per cent, phosphatides 1.3 per cent, polysaccharides, resins, coloring matter, etc., 3.5 per cent, mineral matter 4.4 per cent. Such a plasma mass * is seen to be chiefly a mixture of nucleo- and lipo-proteins, the lipins and pentosans being present in small proportions. Furthermore, nearly all this material would follow the changes in condition of a reversible gel, the insoluble portion being very small.[1]

Proteins may constitute as much as 90 per cent of the dry weight of bacteria. Reproductive elements and chromosomal material seems to be largely made up of nucleo- and lipo-proteins. Notable departures from the above are encountered in the higher plants in which the phospho-lipins, possibly the glyco-lipins, and amino-lipins, and the pentosans or mucilages play a much more important part. Embryonal cell-masses, especially in seedlings and

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* Cumulative colloidal protection may be expected in a mixture like this. See paper on "Colloidal Protection," by J. Alexander, Vol. I of this series. *J. A.*

[1] Lepeschkin, W. W., "Ueber die chemische Zusammensetzung des Protoplasmas des Plasmodiums," *Ber. deut. Bot. Ges.*, 41, 179 (1923).

growing points, are so rich in lipins and lipoidal combinations as to cause Czapek to characterize the living matter as "Lipoplasma." [2]

In the recent notable contribution by Hansteen-Cranner, the peripheral layer of plasmatic substance is regarded as lipoidal, consisting of a disperse phase of hydratable material not soluble in water, in a continuous phase of water-soluble lipoid.[3] This formation is continuous with a fundamental lipoid meshwork of the plasma and strands are supposed to extend through or into the cell-walls. The conclusions thus briefly noted are held to sustain in part the general contention of Overton as to the lipoidal character of the plasmatic membrane. Such an arrangement of lipoids would be one which would permit the passage of both water-soluble and fat-soluble substances. That such a layer does exist was concluded by Boas, who published a preliminary paper to this effect in 1920 and his detailed observations upon which this conclusion was reached in 1921.[4] The experiments were based upon the known reactions of lecithin and cholesterin to neutral salts and to saponin. Measurement of fermentation in yeasts and of the decolorization of cells of higher plants were made the basis for the assertion that a proteinaceous membrane in the cell is highly improbable, and that lipoids are concerned in the exchanges of the cell with the medium. Walter, in confirmation of Biedermann, found that the plasmatic mass of plants is not readily digested by proteoclastic enzymes until the lipoid, which is held to be in a fine state of dispersion, is first extracted by a fat solvent. He holds that his evidence is against the conclusion that the lipoids are localized in a peripheral layer in the cell.[5]

It is to be noted that cytologists retain the conception of a plasmatic membrane as a peripheral layer not separable from the cytoplasm and considered by implication to consist of proteins. Seifriz regards this suppositious membrane as a highly viscous layer about 1 mm. in thickness.[6]

The distribution of the lipins in the cell probably shows a pattern varying with the age of the cell. The importance of this material in permeability will be found greatest when it occupies a peripheral position. A recent attempt to extract the lipoidal material of the Opuntias by Dr. E. B. Working, yielded material which yielded glycerin, fatty acids, choline and phosphoric acids as hydrolysis products. Glyco-lipins were probably included.[7]

The Opuntias have been used extensively in experiments on growth and permeability. Dr. H. A. Spoehr has found the total protein content of active joints to be much lower than the carbohydrates.[8] Of the carbohydrates the pentosans are such a notable proportion that it may be estimated that mucilages are present in an amount two or three times as great as the soluble proteins. These mucilages originate in the protoplast, probably in the vicinity of the nucleus or in whatever part the implied transformations in the polysaccharides take place. It is also seen that in some cases the wall material is broken down

[2] Czapek, F., "Zum Nachweise von Lipoiden, in Pflanzenzellen." *Ber. deut. Bot. Ges.*, 37, 207-216 (1919).
[3] Hansteen-Cranner, B., "Beitrage zur Chemie und Physiologie der Zellwand und der plasmatischen Grenzschichten," *Ber. deut. Bot. Ges.*, 37, 380-391 (1919)
[4] Boas, Fr., "Beitrage zur Kenntniss der Wirkung des Saponins auf die Pflanzliche Zelle," *Ber. deut. Bot. Ges.*, 38, 350-353 (1920). Also, "Untersuchungen ueber die Mitwirkung der Lipoide beim Stoff-Austausch der Pflanzlichen Zell," *Biochem. Z.*, 117, 166-214 (1921).
[5] Walter, H., "Ein Beitrag zur Frage der chemischen Konstitution der Protoplasma," *Biochem. Z.*, 122, 86-99 (1921).
[6] Seifriz, W., "Observations on some physical properties of protoplasm by aid of microdissection," *Ann. Bot.*, 35, 269-296 (1921). [See also paper by W. Seifriz, this volume *J. A*]
[7] Working, E. B., "The isolation of phosphatides from Opuntia discata," *Ann. Report, Lab. for Plant Physiol.*, Carnegie Inst. of Wash., 55, 1923.
[8] Spoehr, H. A., "The carbohydrate economy of the cacti," *Publ. Carnegie Inst. of Wash.*, No. 287, 39 and 40, 1919.

and forms a slime, which however would be of minor importance in the present connection.[9]

The view that protoplasm is an emulsion can not be accepted as a comprehensive conception of its condition without many modifications. Recent evidence tends to show that gelatin as a representative of the proteins takes the form of a two-phase liquid-solid system. The considerations upon which the solid particles are taken to be minute spherites of solid material to which water is held by adsorption have been recently presented by Dr. Bradford.[10] These spherites may coalesce in various forms including those of visible crystals. A similar arrangement is found in agar-agar gels.

A liquid-solid arrangement of such gels seems to have been originally proposed by Procter, who took a gelatin jelly to be a network of amino-acid chains, adhering by the acidic and basic terminals.[11] Water, with perhaps some unattached molecules, fills the interstices of such a system.*

The intermixture of the pentosanic and albuminous components includes possibilities not to be described here. The gelation of each would take place in such manner as to arrange the particles with the most active groups inward or united with the formation of lattices as described. If the active groups were ouward, the play of intermolecular forces would result in a different set of adsorptions and characteristic system of charges.

Some of this variability may be reproduced in the blending and cooling of agar-gelatin-lecithin mixtures. Aggregation and mutual precipitation effects are to be seen. Dissociated groups having adsorbed ions from the solutions, may exhibit Brownian movements. The plasmatic mass may thus include tracts in which the fibrillar or crystalline structure is simple, with ionized substances in water filling the interstices, a true gel; other portions may be aggregations and others fluid with free moving particles.

The heterogeneous mass may enclose and hold the lipins, lipo-proteins, glyco-lipins, etc., and other substances in the form of minute rounded liquid particles, making some simulation of an emulsion.

It is in this field of ultra-microscopic magnitudes that the work of the colloidal physicist and the cytologist must meet to solve the fundamental problems of protoplasmic mechanics.[12]

The reactions of the plasmatic mass to water under the influence of the substances produced by its resident metabolism and as affected by entering ions, may be primarily studied by experiments with its separate constituents. These may be combined in the laboratory in such manner as to give a widely

* Stewart, E. G., "Mucilage or slime formation in the cacti," *Bull. Torr. Botan. Club.* 46, 157-166 (1919).

Smith, E. L., "The histology of certain orchids with reference to mucilage secretion and crystal formation."

Lloyd, F. E., "The colloidal properties of protoplasm-imbibition in relation to growth," *Trans. Roy. Soc. Canada*, III, 11, 133-140 (1917). [See also paper by F. E. Lloyd, this volume. *J. A.*]

[10] Bradford, S. C., "The nature of gels," *Nature*, 111, 200-202 (1923). [See also paper by S. C. Bradford in Vol. I this series. *J. A.*]

[11] Procter, H. R., "The structure of organic jellies," Proc. Seventh Internat. Congress of Applied Chemistry, London (1909).

Procter, H. R., "The structure of elastic jellies," Physics and Chemistry of Colloids. Faraday Soc. London, see 40 (1921).

Wilson, J. A., "The swelling of protein jellies," Colloid 'Symposium Monograph, Madison, Wisconsin, June, 1923.

* Several papers in Vol. I of this series, take a different view; e.g. papers by Buchner and Kopaczewski. *J. A.*

[12] Kite, G. L., "The physical properties of the protoplasm of certain animal and plant cells," *Am. J. Physiol.*, 32, 146 (1913).

Seifriz, Wm., "Viscosity values of protoplasm as determined by the aid of microdissection," *Bot. Gaz.*, 70, 360 (1920)

"Phase reversal in protoplasm and in emulsions," *Science.* 57, 694 (1923).

Wilson, E. B., "The physical basis of life," Yale Univ. Press, New Haven, Conn., 1923.

embracing pattern of reactions parallel to those of living cell-masses. The alterations most readily measurable are those of dimension or volume. Changes of this kind may be made with the auxograph designed for this purpose (Fig. 1).

A compound lever is carried by a vertical post adjustable as to height by a rack and pinion. A vertical swinging arm, the lower end sheathed in
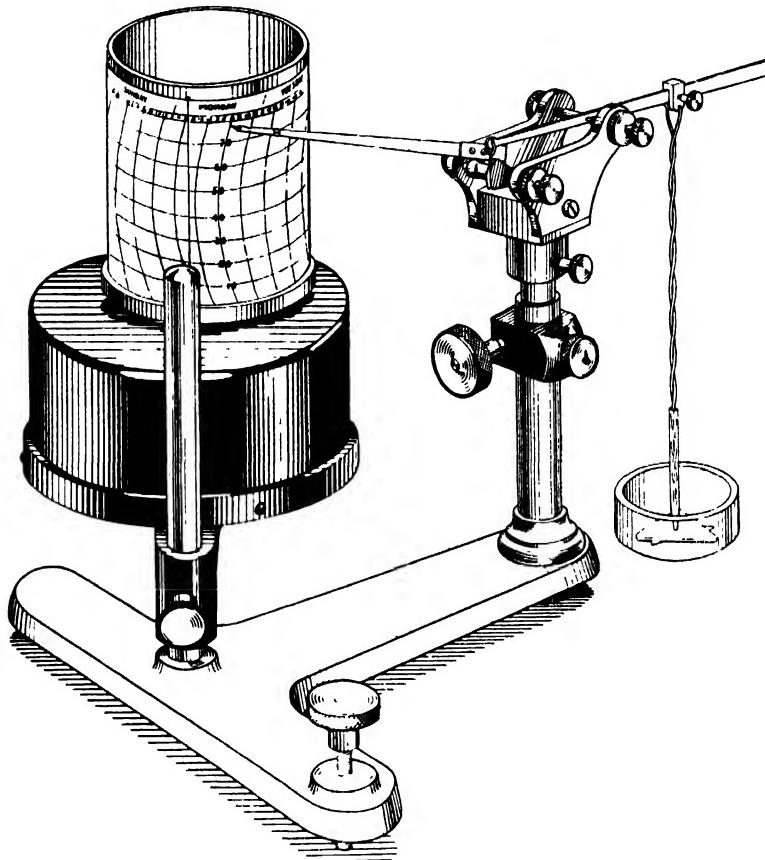
Fig. 1.—Auxograph.

glass tubing, is attached to the free end of the lever. Trios of sections of dried colloids are placed in a small stender dish and a thin triangular plate of glass is laid over them. This glass plate is perforated at the center to make a secure bearing for the tip of the swinging arm, and over each of the three sections to permit access of the solutions used. When such solutions are poured into the dish, the swelling of the colloids causes the longer arm carrying a pen to trace a record on a sheet ruled horizontally to millimeters and vertically to two-hour periods. The record sheet is carried on a cylinder rotated by an 8-day clock. Approximate satisfaction of the colloids may
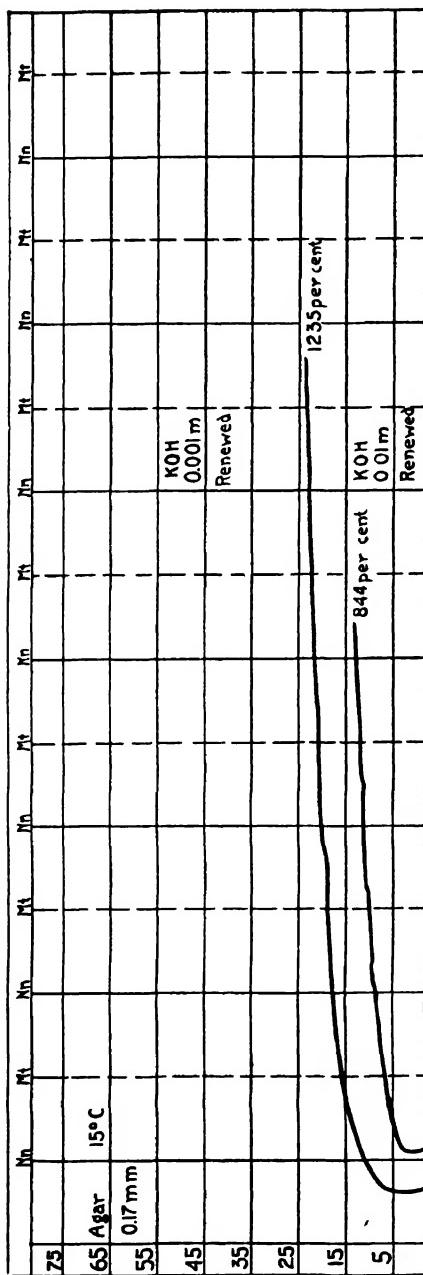
Fig. 2.—Auxographic records of swelling of agar plates in KOH solutions, which were renewed every 12 hours.

(from *Botanical Gazette*, Vol. 70).

require three to eight days, or as long as a fortnight in some cases at 15-18° C.*

The agar used in securing the measurements given below was prepared by the following method: A good grade of commercial agar was dissolved in distilled water and jacketed with superheated steam. The viscous solution produced thereby was filtered clear through a thick mass of steam-jacketed paper pulp, under diminished pressure. The clear solution of agar was dialyzed for about 10 days in a steam-jacketed bath containing running distilled water. After removal of diffusible carbohydrates and salts by dialysis through parchment paper of the quality usually employed in the serum industry, the clear solution was slowly poured in a fine stream or spray into 10 times its volume of neutral acetone. The agar was precipitated in the form of fine shreds. It was subsequently extracted with hot absolute acetone, absolute alcohol, and absolute ether. The final product was ground to a granular powder in a porcelain ball-mill with porcelain balls. The resulting preparation contains only a trace of nitrogen and a trace of ash.

Samples of this agar made up as a 2.5 per cent solution with distilled water had a light brown tinge, dissolving completely within an hour at about 100° C. It was found that the material made up as a 0.75 per cent solution, when poured into a test-tube formed a "slant" which kept in place when the tube was set in an upright position at 15° C. for 2 weeks. Solutions near this concentration showed pH $= 6.5$. Homogeneous plates were secured by pouring layers 5 to 6 mm. in thickness on glass plates, which dried to a thickness of 0.12 to 0.2 mm. Sections cut from these plates were hydrated in various solutions, and the amount of swelling measured by the auxograph. The increases given below are in percentages of thickness of dry sections hydrated at 15-16° C.

The coefficient of increase of plates made at different times, of different thicknesses, varies so that strict comparisons are possible only among sections from one plate hydrated simultaneously. This necessitated the use of a battery of auxographs.[13]

The hydrating solution was ordinarily renewed every 12 or 24 hours. In the later stages of swelling this renewal was followed by an abrupt expansion of the colloid. All of the increase would be retained in the earlier stages of swelling. Later some of the increase would be lost. The loss increased as satisfaction was approached (Fig. 2).

The swelling reactions of agar in the presence of acids, neutral salts, and hydroxides of the common bases are illustrated by the measurements in Table 1.

It is evident that swelling in the neutral chlorides is greater in dilute solutions than in water. Such excessive increase begins in KCl and NaCl at some concentration between 0.01M and 0.001M. Excessive increase is not seen with the bivalent cations Mg and Ca until a dilution of 0.0001M is reached.

The increases in HCl and in the hydroxides show maxima on both sides of neutrality. On the acid side at about 0.0001 N, pH 3.01, at 0.001 N, pH 11, in the hydroxides of the univalent bases, and at 0.0001 N with the bivalent bases. These maxima form part of the facts upon which the cell is treated as an ampholytoid by some writers.[14] Furthermore, the methods em-

* This fact may seriously affect some data based on "over-night" treatment. *J. A.*
[13] MacDougal, D. T. and Spoehr, H. A., "The swelling of agar in solutions of amino-acids and related compounds," *Bot. Gaz.*, 70, 268 (1920).
[14] Robbins, W. J., "An isoelectric point for plant tissue and its significance," *Am. J. Bot.*, 10, 412 (1923).

TABLE 1. *Increases of Sections of Agar in Chlorides and Hydroxides.*

Agar in Chlorides.*

	HCl	KCl	NaCl	MgCl₂	CaCl₂
0.01N	700	1305	1145	825	705 at 0.01M
0.001N	1600	2900	2680	1590	1190 at 0.001M
0.0001N	2100	2645	2975	2315	2160 at 0.0001M
					Water 1900

Agar in Hydroxides.*

LiOH	NaOH	RbOH	KOH	Ba(OH)₂	Ca(OH)₂	Sr(OH)₂
1820	1645	1635	1535	900	860	815 at 0.01N
3430	3430	3500	3430	1145	1220	1565 at 0.001N
				2430	3200	2565 at 0.0001N
				2400	3200	2665 at 0.00001N
						Water 3000

* Swellings in excess of the volume in water are in **bold face**.

ployed by some workers do not show the excessive swelling of agar in acid solutions.[15]

The swelling of gelatin reaches a maximum in acid, and in neutral salts at maxima determined by the pH in one case and by the formation of a salt with the cation in the other.

The increases in neutral salts are illustrated by the following data obtained by auxographic measurements.

TABLE 2. *Swelling of Gelatin in Neutral Salts.*

KCl		CaCl₂
2100		670 at 0.01M
1400		1596 at 0.001M
1160		1000 at 0.0001M
	Water 910	

The principal changes in volume of a plasmatic mass are to be attributed to the hydration capacity of the mixture of pentosans and albumin derivatives present. The maximum under acid or alkaline conditions will be the resultant of several factors including the structure of the compounds, the proportions of the two main components, and the electrolytes present.

This is illustrated by the swelling reactions of two mixtures of agar and gelatin as measured by the auxograph (Table 2a).[16]

It is to be seen that a mixture of pentosans and gelatin has a much higher hydration capacity when the pentosans form more than half of the total volume.

Variations in the rate of increase and departures from isodiametric expansion have distinct importance to the physiologist. The last-named feature

[15] Fairbrother, F., and Mastin, H., "The swelling of agar-agar," *Trans. Faraday Soc.*, **123**, 1412 (1923).

[16] MacDougal, D. T., "Water deficit and the action of vitamines, amino-compounds and salts on hydration," *Am. J. Bot.*, **8**, 296 (1921).

"Auxographic measurements of swelling of biocolloids and of plants," *Bot. Gaz.*, **70**, 126 (1920).

TABLE 2a. *Swelling of Mixture, Agar 2 Parts, Gelatin 3 Parts.**

KCl	NaCl	Bal. Sol.	CaCl₂
880	965	$\left\{ \begin{array}{l} \text{NaCl} \\ \text{CaCl}_2 \end{array} \text{at } 0.01\text{M} \right\}$	740 at 0.01M
1010			940 at 0.001M
1850		1235	**1430** at 0.0001M

Water 1420

*Swelling of Mixture, Agar 3 Parts, Gelatin 2 Parts.**

KCl	Bal. Sol.	CaCl₂
2015	$\left\{ \begin{array}{l} \text{NaCl} \\ \text{CaCl}_2 \end{array} \text{at } 0.01\text{M} \right\}$	1140 at 0.01M
2270		1220 at 0.001M
2640	1235	2268 at 0.0001M

Water 2330

* Swellings in excess of volume in water are given in **bold face.**
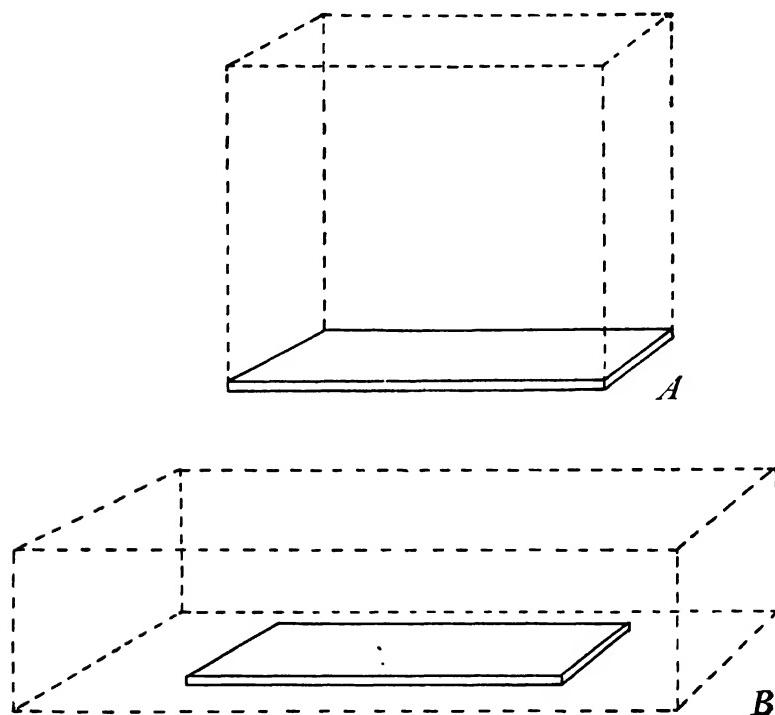
FIG. 3.—Initial and hydrated forms of agar and of gelatin plates.
(from *Botanical Gazette*, Vol. 70).

is illustrated by Figure 3 in which the initial and hydrated forms of sections of plates of agar and of gelatin are given.

Certain transient and permanent variations in biocolloids which were first
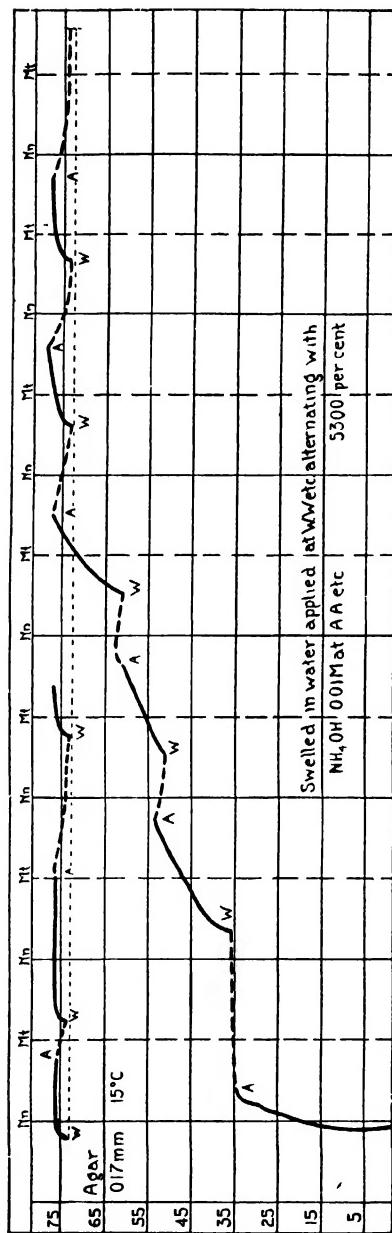
FIG. 4—Auxographic records of swelling agar.

(from *Botanical Gazette*, Vol. 70).

measured by MacDougal and Spoehr have some possible importance in physiology.[17]

In hydrations of sections under the auxograph three sections with a total dry volume of 3 to 5 cu.mm. were placed in dishes with a capacity for about 30 cc. Solutions were renewed at intervals of 12 or 24 hours.

Agar swelled in NH_4OH to an amount about one-fourth that in water. If sections which had been approximately satisfied in water, and this was replaced by NH_4OH at 0.01 N, a further expansion occurred, although a shrinkage might have been expected. It may be suggested in this case that the water of hydration in the agar acted to dilute the ammonia, and at lower concentrations, as noted at 0.001 N ammonia hydroxide causes a hydration in excess of that in water. The total swelling in such alternation of water and NH_4OH was found to be 5300 per cent, resulting in a firm jelly in which the solids formed less than 2 per cent of the dry weight (Fig. 4).

The cumulated swelling of alternating immersion in water and in glycocoll at 0.01M is a different case. Such a solution has an acidity of pH 6.2, slightly in excess of that of the agar at 0.75 per cent dry weight, which was pH 6.5. Such a solution would include the internal salt of the glycocoll, some of its ions in a hydrated form, as well as other possible combinations (Fig. 5). Agar hydrated in a series of graded solutions [18] shows the greatest swelling in the region of 0.01M glycocoll, but high values are obtained in concentrations as low as 0.002M and even 0.0004. Now when sections are alternately immersed in glycocoll and water, the cumulated increase results in a volume which was as 191 compared to water at 100 or as to 123 as to the highest recorded value in glycocoll alone. The highest swelling in ammonium hydroxide recorded was 115. These facts take on unusual significance when it is recalled that the colloids of the active cell are subjected to incessant changes in the matter of infiltrating substances, and that the results of direct tests of hydration increases may be considered as far below the maximum which would be produced by the alternating action of substances in solution. MacDougal and Spoehr in 1920 concluded that

"From a consideration of the relative effects of various hydroxides it would appear that something analogous to salt formation and subsequent hydrolysis of this compound may be involved. Ammonium, as a weak base united with agar, an exceedingly weak acid, would form a salt which would very easily be hydrolyzed. Under conditions in which this hydrolysis is suppressed by the presence of a common ion (NH_4), the excessive swelling does not take place. Thus when dried agar plates are allowed to swell by alternating solutions of 0.001N, NH_4OH, and NH_4Cl the total swelling does not exceed that attained in water, but actually falls somewhat below that value."

Fairbrother and Mastin [19] later reported that disks of agar when placed in NaOH 0.01 N and water alternatively swelled more than either separately. These writers had concluded that agar is chiefly the acid salt of an acid sulfuric ester which becomes ionized in the gel as well as in the solid state. Sodium was found to have replaced calcium in the sections alternatively placed in NaOH and in water.

[17]MacDougal, D. T. and Spoehr, H. A., "Swelling of agar in solutions of amino acids and related compounds," *Bot. Gaz.*, **70**, 268 (1920) especially 276-278 and Fig. 6.
[18] MacDougal, D. T. and Spoehr, H. A., "The swelling of agar in solutions of amino-acids and related compounds," *Bot. Gaz.*, **70**, 268 (1920).
[19] Fairbrother, F. and Mastin, H., "The swelling of agar-agar," *Trans. Faraday Soc.*, **123** 1412 (1923).
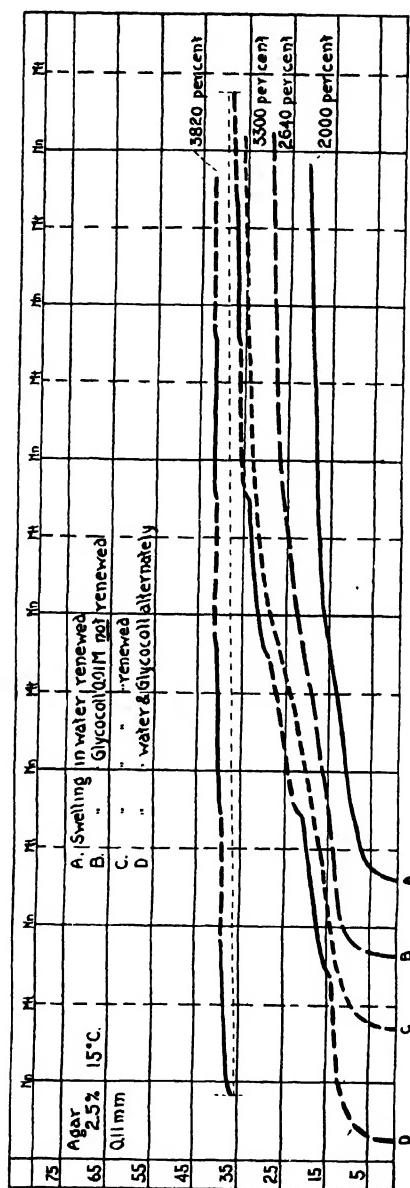
FIG. 5.—Auxographic records of swelling agar.

(from *Botanical Gazette*, Vol. 70).

PERMEABILITY

The arrängement of the colloids of the plant-cell in the wall and plasmatic layer in such manner as to constitute a complex unstable membrane, is a matter of great importance as it is through this material that all substances pass into and out of the plant. A young cell is a dense mass of protoplasm in its earlier stages. Enlargement of such masses is by the formation or adduction of new material and by hydration or swelling. By the time a definite bounding layer or wall has become apparent, syneretic cavities or vacuoles appear. Sugars, amino-acids and salts accumulate in these vacuoles, and
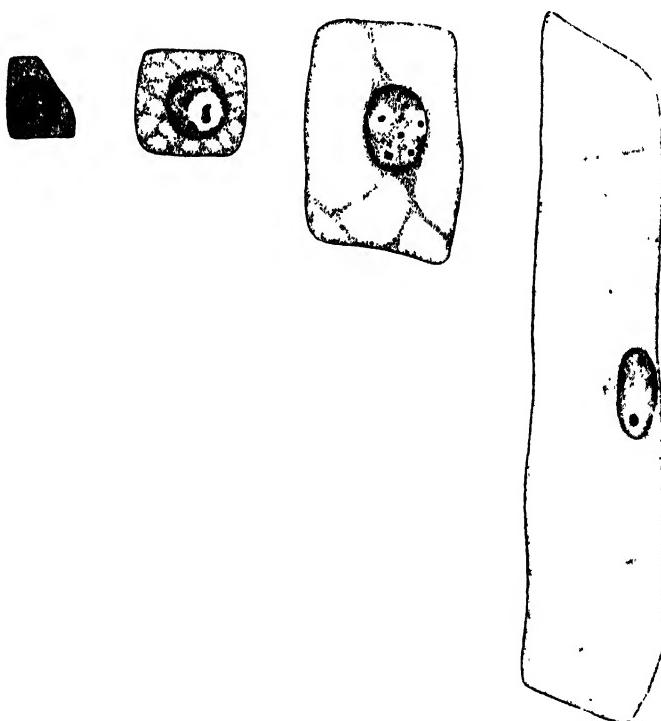
FIG. 6.—Arrangement of cell components.

by their osmotic action turgor pressure is set up, which is responsible for the distention of the cell to mature size. Nine-tenths or more of the volume of the cell is thus due to other distensive action of the substances in the cavities of the vacuoles into which almost any soluble substance may pass. The arrangement of the cell-components concerned is of prime interest (Fig. 6).

The outside wall of a cell in the distended stage has a skeletal structure of cellulose, which will swell slightly in water but is insoluble to cell solutions, except under the action of enzymes. A mixture of mannosans, glucosans, and pectins, all liquefiable in water, occupy the meshes of the cellulose skeleton. Lipins or phosphatides have been shown by Czapek to be a constituent of such high proportion in the plant cell as to give living material an essential

lipoidal structure according to this writer. Hansteen-Cranner has found that the lipins accumulate in the peripheral part of the cell and that processes or strands of this material extend into the walls and also into the gelatinous mass of the protoplasm. The other constituents of the mass would include pentosans, and albumins and albuminous derivatives, as well as various compounds or salts of these substances.[20]

Permeability may be defined as the condition of the colloidal layers which permits the passage of water, of electrolytes, or of particles in suspension or solution, into and out of a cell. As permeability lessens, it finally reaches a stage in which only water or the more mobile ions may pass through the layers. In this stage the osmotic action of the vacuolar contents or cell sap is highest. As permeability increases and the ions of the common salts of sodium, potassium, magnesium and calcium, as well as the particles or molecules of sugar and organic substances may traverse the layers more readily, the osmotic action of the cell is lessened.

Determinations of permeability have been made by a wide variety of methods, including the study of visible and microchemical changes in the cell, analysis of sap and of the medium, measurement of hydrogen-ion concentration in the sap and in the external solution, measurements of the electrical conductivity of the cell and of the medium, estimations of metabolism, plasmolysis, plasmometry, measurements of tissue tensions, and of diffusion, and finally by measurements of changes in weight and volume of living cell-masses induced by the entrance or loss of water and of other substances.[21] The inadequacy of any one of these methods taken alone, the number of workers engaged, the lack of correlation of the literature and the general great interest in the subject, have resulted in a voluminous literature, which has been recently reviewed comprehensively by Stiles.[22]

The ions of the common substances have been found to exert individual and characteristic effects on the permeability of the unstable membrane composed of the wall and plasmatic layer of the plant-cell. This effect has been referred to valence, to the relative density of the charges carried, and to the mass or mobility of the ions by various writers.

The complex membrane which is constituted of the plasmatic layer and the wall of a plant cell is in a continual state of alteration by loss and accretion of material. The molecular groups may be undergoing continuous changes in arrangement, as has been previously described. The play of adsorption phenomena of a reversible type in such a mass is much more complicated than that which takes place on the proteins alone. It is therefore not possible to accept any formula derived from the action of a durable membrane such as those of parchment and collodion as adequate for the analysis of permeability in a living or artificial cell.[23]

[20] Hansteen-Cranner, B., "Beiträge zur Chemie und Physologie der Zellwand und der plasmatischen Grenzschichten," *Ber. deut Bot Ges*, 37, 380-391 (1919).
Czapek, F., "Zum Nachweise von Lipoiden in Pflanzensellen," *Ber. deut. Bot. Ges.*, 37, 207-216 (1919).
Walter, H., "Ein Beitrag zur Frage der chemischen Konstitution der Protoplasma," *Biochem. Z.*, 122, 86-99 (1921).
[21] Osterhout, W. J. V., "A dynamical theory of antagonism," *Proc. Am. Phil. Soc.*, 55, 553 (1916).
Raber, O., "Permeability of the cell to electrolytes," *Bot. Gaz.*, 75, 298 (1923).
Loeb, J., "Hydrophilic and hydrophobic colloids and the influence of electrolytes on membrane potentials and cataphoretic potentials," *J. Gen. Physiol.*, 6, 307 (1924).
[22] Stiles, W, "Permeability," A comprehensive review, citing 817 papers running through numbers of *The New Phytologist* from June 30, 1921 to December, 1923.
[23] Loeb, J., "The origin of the electrical charges of colloidal particles and of living tissues," *J. Gen. Physiol.*, 4, 351 (1922).
Gordon, Neil E., "Gels and theory of adsorption," *Science*, 57, 495 (1923).

The relations of electrolytes to pentosans and to cells may be most usefully expressed on the basis of a series in which the ions are arranged according to their translational velocities, which may be considered as a straight line function of the density of the charge carried. This is not to be taken as an adequate statement of causal relation, but as a parallel from which departure

may be made in an analysis of the mechanism of permeability. This has been most clearly brought out in the work of Kahho in the generalization that every ion lessens the permeability of plant cells for ions preceding it in the series and increases it for the ions to the right in accordance with the rule that: *"Bases carrying a charge of opposite sign to that of a colloid, exert a dehydrating or aggregating effect proportional to the unit charges which they carry."* By measurements of the reactions of roots of lupine to neutral salts in hypotonic solutions, he confirms the series as to permeability of cations which runs $K > Na > Li > Mg > Ba > Ca$, in which the greatest penetrability is shown by potassium and the least by calcium. It is also seen that the interferences are such that each cation is retarded by those to its right, and to a degree proportionate to its distance to the right, and that the greatest retardation is by the cations which show the greatest coagulating action on colloids. The cations which have the least coagulating action on colloids penetrate most rapidly. The anions retard the colloidal or coagulative action of the cations in a series, citrate $<$ sulfate $<$ tartrate $<$ Cl $<$ NO_3 $<$ Br $<$ I, in which the effect is least with the citrate and most with the iodine. That is, each cation has the greatest effect when combined with the citrate and least with the iodine. As a fur-

Fig. 7.—Imitation "cell."

ther consequence, the citrates have the least *penetrability* and the salts of iodine greatest.[24]

As the effects of ions on cells is clearly additive or resultant of the electrical disturbances set up in the different components, which widen or close the meshwork concerned in permeability, an artificial cell was designed which could be varied to present differential effects of its components and of entering electrolytes expressed in such manner that direct comparisons with the action of living cells was possible.[25]

This cell in its complete form is constructed as follows (Fig. 7):

[24] Kahho, H., "Zur Kenntniss der Neutralsalzwirkung auf das Pflanzenplasma," *Biochem. Z.*, **120**, 125 (1921).

"Ein Beitrag zur Permeabilität des Pflanzenprotoplasmas für Neutralsalze," *Biochem. Z.*, **123**, 284-303 (1921)

[25] MacDougal, D. T., "The probable rôle of lipoids in growth," *Proc. Am. Phil. Soc.*, **41**, 33 (1922).

"Permeability and the increase in volume of living and artificial cells," *Proc. Am. Phil. Soc.*, **72**, 6 (1923).
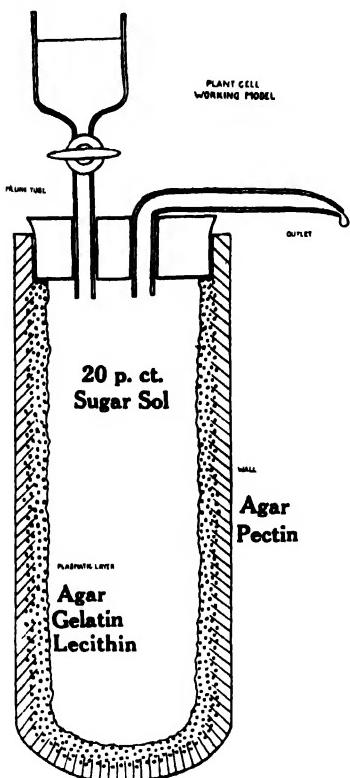
An extraction thimble, double thickness, 33 × 80 mm. (Whatman or· Schleicher and Schüll) is fitted with a ground glass neck 2 cm. deep, fitting snugly in place, and a suitable rubber band is put around the thimble near the top or open end. The thimble is now dipped in a 5 per cent solution of agar, liquefied at 80° C., and set, open end down, on a glass plate to allow the jelly to cool and harden. A half hour later the thimble is thrust, closed end downward, into a warm solution of pure pectin in the same manner as with the agar, none of either jelly being allowed to get inside the thimble.
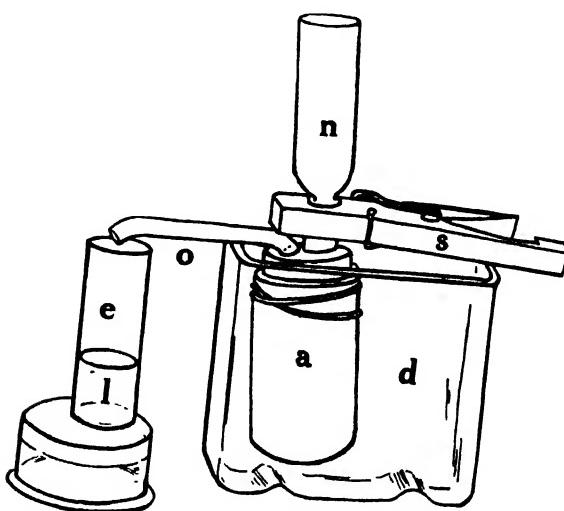
Fig. 8.—Imitation "cell," ready for use.

It is again set, open end down, to cool. After this the preparation is placed in ethyl alcohol (some denatured preparations are suitable) for 5 to 10 minutes, then again set, open end downward, to drain and allow the alcohol to evaporate.

After 2 to 4 hours, while the thimble is still moist, it is filled with an alcohol emulsion or aqueous solution of lecithin, which is allowed to remain until the liquid shows on the outside. This carries the lipin into the interstices of the pentosans of the wall. After draining for two hours, 10 cc. of a warm mixture of agar and gelatin or agar and some albumins is poured into the thimble, which is turned in the hand until the mixture has cooled to a uniform layer, which locks into the inner layers of the mesh of the thimble.

A No. 6 rubber stopped fitted with a filling funnel and a horizontally bent outlet, is now inserted in the glass collar and the cell is ready for use immediately (Fig. 8).

In nearly all of the experiments described below, 20 per cent sugar solution has been used as "cell-sap" and about 50 cc. will fill the cell as prepared. Larger or smaller thimbles may be used in the construction of the cell.

The cell is supported in an upright position in a **glass vessel** which contains 7 or 8 times as much immersion solution as the contents of the cell.

Within an hour or two the cell will show endosmose and an extrusion of water from the outlet tube, if some such pair as cell-content of 20 per cent sugar solution and an immersion liquid of KCl 0.01M is arranged. Endosmose of one series of cells is shown in the following table:

Table 3. *Endosmose of Agar-Pectin-Gelatin-Lecithin Cells Filled with 20 per Cent Sugar Solution at 12-18° C.*

	KCl	NaCl	CaCl₂	Seawater	Water
Immersion liquids	0.01M	0.01M	0.07M	0.01M for Na	
Total for 2 cells for 70 hours..	22.2	25.7	27.6	22.6	24.1

Measurement of changes in acidity, of the changes in conductivity, and of the variations in the salts of the contents and immersion, may also be made. It is to be noted that the amount of endosmose is inverse to the permeability within the range of biological interest. Comparative results obtained by such cells are given below:

TABLE 4.

Absorption of Water from Solutions by Agar-Pectin-Lecithin Cells, Contents 20 per Cent Sugar Solution.

Seawater < NaCl < Water < KCl < CaCl₂ at 0.01M

Absorption of Water from Solutions by Agar-Pectin-Lecithin-Gelatin Cells, Contents 20 per Cent Sugar Solution.

KCl < Seawater < NaCl < Water < CaCl₂ at 0.01M

The two sets of results show the displacement of K in the series by the absence or presence of gelatin in the cell, making a point of departure for the study of the accumulation of this element in the cell sap. These results are to be compared to the measurements of the swelling of living and dead cells, in which endosmose results in an enlargement which may be recorded by the auxograph. K and Na are seen to be shifted in the change from living to dead cells (Table 5).

TABLE 5.

Proportionate Total Swelling of Dried Sections of Opuntia.

KCl 310   Seawater 310   NaCl 280   CaCl₂ 275 at 0.01M,   Water 255

Proportionate Total Swelling of Living Sections of Opuntia.

NaCl 140   KCl 145   CaCl₂ 160 at 0.01M,   Water 160.

Time Necessary for Satisfaction of Living Sections of Opuntia.

KCl 30 hours,   CaCl₂ 36 hours,   NaCl 48 hours.

The amounts of the various ions taken up by living cells as determined by various authors using different methods are given in Table 6.

TABLE 6.

Adsorption of Cations by Seeds.

K < Na < Mg, etc.   Direct analysis
    Ascribed to migrational velocities.   Slosson (1899).

Adsorption of Ions by Spirogyra.

K > Na > Ca   NO₃ > Cl   Ruhland (1909).

Adsorption of Ions from Neutral Salts, by Rheo.

                          Measured by plasmolysis.
                                     Fitting (1915).

(K, Na) > Li > Mg > (Ca, Ba)   NO₃ > Cl > SO₄

Adsorption of Ions by Cicer arietinum, Vicia faba, Valonia utricularis,
Ulva lactuca and Yeast.
                                      Pantanelli (1915).

Adsorption of calcium is less than that of potassium in higher plants,
while this is reversed in lower plants.

Adsorption of Cations by Roots of Lupine, Palisade Cells of Acer
platanoides and Salix babylonica.
                                      Troendle (1918).

Rb > K > Na > Li > Mg > Ba > Sr > Ca   NO₃ > Cl > SO₄

Adsorption of Ions by Carrots and Potatoes.

                                    Conductivity tests.
                                    Stiles and Kidd (1918).
KCl > NaCl > LiCl > (CaCl₂, MgCl₂) > NO₃ > Cl > SO₄

Permeability of Roots of Lupine to Neutral Salts.

                      Determined by plasmolysis and contraction.
                                    Kahho (1921).
Rb > K > Na > Li > Mg > Ba > Sr > Ca

The results are to be compared to the measurements of absorption and penetration of artificial cells as measured by conductivity tests given in Table 7.

TABLE 7.

Adsorption of Ions from Immersion Liquid by Agar-Pectin-Gelatin-Lecithin Cells,
Contents 20 per Cent Sugar Solution.
NaCl < KCl < Seawater < CaCl₂   at 0.01M

Penetration of Agar-Pectin-Gelatin-Lecithin Cells,
Contents 20 per Cent Sugar Solution.
KCl > CaCl₂ > NaCl > Seawater   at 0.01M

The errors due to the presence of sugars and other organic compounds which may arise in measurements of conductivities of such electrolytic solutions, have not been determined for these experiments.[26]

[26] Stiles, W. and Kidd F., "The influence of external concentration on the position and equilibrium attained in the intake of salts by plant cells," *Proc. Roy. Soc.*, **B90**, 448 (1919).

The retarding effects of anions on the action of cations in penetrating cells is illustrated by the results in Table 8.

TABLE 8.

Retarding Effects of Anions on Cations.

Measurement of length of apical
parts of young roots—Kahho (1921).

Citrate $<$ sulfate $<$ Tartrate $<$ Cl $<$ NO$_3$ $<$ Br $<$ I

Effects of the Salts of Sodium in Increasing Permeability of Laminaria.

Conductivity tests.
Raber (1920).

SCN $<$ I $<$ Br $<$ NO$_3$ $<$ Cl $<$ Acetate $<$ Sulfate $<$ Tartrate $<$ Phosphate $<$ Citrate

The duplication and confirmation of these results with variations produced by changes in cell construction are shown in Table 9.

TABLE 9.

Effect of Anions of Sodium Salts on Permeability of Agar-Pectin-Gelatin and Agar-Pectin-Gelatin-Lecithin Cells, Contents 20 per Cent Sugar Solution.

Water $<$ Nitrate $<$ Chloride $<$ Sulfate   at 0.01M

Effect of Anions of Potassium Salts on Permeability of Agar-Pectin-Gelatin Cells, Contents 20 per Cent Sugar Solution.

Iodide $<$ Nitrate $<$ Chloride $<$ Sulfate   at 0.01M

Similar relative effects of anions on the hydration of agar are illustrated by the auxographic measurements in Table 10.

TABLE 10. *Proportionate Increases of Agar in Sulfates and Nitrates.*

	Na		K	
	0.01M	0.0001M	0.01M	0.0001M
Sulfates	740	2215	710	2515
Nitrates	990	2720	827	2910
Water	2400	....	2560	....

The nitrates cause a hydration greater than that by the sulfates at all concentrations, the increase in the more attenuated solutions of both being greater than that in water. It is also seen that swelling is greater in the salts of K than in Na. Increase in hydration implies increased permeability. The high degree of correlation between these effects and ionic mobilities is apparent. Current determinations of these are given for convenience in Table 11.

TABLE 11. *Ionic Mobilities.*

H 318,   Rb 67.5,   K 64.6,   Ba 55,   Ca 51,   Sr 51,   Na 43.6,
Mg 45,   Li 33.4,   SO$_4$ 68,   Br 67,   I 66.5,   Cl 65.5,   NO$_3$ 61.7
at 18° C.

The origination of electrical charges on colloidal particles, the possible unions which include the entire range of absorption and the composition and arrangement of the wall and plasmatic layers are also to be considered.

Colloidal Aspects of Botany

By Prof. Francis E. Lloyd,

McGill University, Montreal, Quebec, Canada

Earlier speculation on the origin of living substance considered the problem as a purely chemical one. It appears clear now, however, that the substratum for the process of organization must have been colloidal, affording a concatenation of the substances whose interplay releases or stores up energy [Verworn and Lee, 1899]. For organization implies more than chemical transformation; it implies also localization made possible by the segregation of interacting materials by surfaces, so that each separated locus is, as it were, a small laboratory on its own [Alsberg, 1921]. It implies also direction and correlation of activity; that is, the building up of a structure (morphogenesis) which realizes the mechanism necessary.

The chemical problem consists in the search for spontaneous (catalysed) reactions of a colloidal nature which can afford energy and, more especially, for photochemical reactions which can go on spontaneously and absorb energy, e.g., the reduction of CO_2 to formaldehyde, of nitrates to nitrites. The broader physical problem consists in finding a colloidal complex to serve as a nidus to hold together the chemical progeny, and which would serve as a beginning for a primeval cell. Such a cell, like the colloidal complex which preceded it, must have been capable of synthesizing carbon and nitrogen compounds, deriving energy from sunlight and rendering it latent. Such a system would be an inorganic colloid capable of adsorbing organic materials. The inefficient transformers or catalysts such as iron which make use only of light of short wave lengths [Moore, 1914, 1918; Moore and Webster, 1920; Baudisch, 1923], became supplanted during the course of development by substances which could employ longer wave lengths. Such a substance is chlorophyll and it is significant that iron, while not entering into the composition of the chlorophyll molecule, is necessary, together with light, for its elaboration. It is therefore an attractive idea that chlorophyll is the first product of photosynthesis [Moore and Webster, 1920], a view which has a number of times been suggested, but has not been free from objection.[1] The possible intercalary position of the pyrrol nucleus, for the formation of which iron again is a catalyser, is an indicated step in the course of events [Oddo and Polacci, 1920].

The interfaces between the catalysing apparatus and the plasma afford the scene for possible electronic action [Gibson, 1908], and this suggestion has received some support from the work of H. H. Dixon and N. G. Ball [1922]. Whether the exchanges between chlorophyll or other catalysts of this sort, e.g., phycocyanin, phycoerythrin, if such they be, and the contiguous surfaces are electronic or not, it seems certain that the scene of action is in the surface of the protective hydrophile or lyophile colloids. Their protective

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[1] Pfeffer's "Physiology of Plants," p. 320.

function may perhaps be taken to involve also a regulatory one [Wurmser, 1920], insuring a slower and more effective breaking down of the pigment, and enabling the organism to seize upon conditions for synthesising chlorophyll which would otherwise be unfavorable. Wurmser found that lyophile colloids (gelatin, egg albumin, gum arabic) exert upon suspensions of chlorophyll a protective action against light. The idea that the surface of the water phase facing the chlorophyll is the theatre of action, receives support from the fact that very minute amounts of surface active substances can affect carbon assimilation [Warburg, 1919]. After finding evidence in support of the lipoid-dissolved state of chlorophyll, Stern [1920] specifies that the *water/chlorophyll-in-lipoid* surface is this theatre. It will be convenient presently to consider the relation of the light-active pigments.

PIGMENTS

Some pigments are water soluble, and are found in the sap of the cell (anthocyanins: *Beta, Allium,* etc.) giving a general hue to the tissues. They can be used as indicators in experimental work on the physiology of the cell. Whether they are always in molecular solution is questionable. They may be combined in some way with other substances which prevent their outward diffusion, and this may not be wholly a matter of permeability.

Certain other water soluble pigments are probably of importance as catalysts, and these are fluorescent. Phycocyanin, phycoerythrin are examples. The former is probably held in minute vacuoles in large numbers [Lloyd, 1923]; the latter is sometimes associated with chlorophyll in the chloroplast [Osterhout, 1916]. The precise mode of occurrence of phycoerythrin is not understood. J. Reinke [1886] believed that it is compounded with the chlorophyll, the loose bonding being set free on death. Osterhout's observations that this pigment is set free when the cell is merely touched, and that, on reaching nearby chloroplasts by diffusion, more pigment is there set free, is against Reinke's view, though it does not exclude its possibility. The relation is an exceedingly delicate one, and comparable to that of hemoglobin in the erythrocyte.

The other pigments which are not water soluble include the carotins, lycopersicin, xanthophylls and chlorophylls, diatomin, etc. The way in which such of these pigments as occur in the plastids exist there, has been under much controversy. It has been held that chlorophyll in the chloroplast is in a colloidal condition [Herlizska, 1912, Iwanowski, 1913, and others], and contrarily that it is in true solution in a fat or oil [Wager, 1905; Kerner, 1893], or, more specifically, in the lipoid of the chloroplast [Liebaldt, 1913, Stern, 1920, 1921]. Others have voiced somewhat equivocal views. The solubility of chlorophyll in fats and oils predisposes toward the argument that it is similarly held in the chloroplast, and this predisposition is strengthened by the idea that chlorophyll has been held not to be fluorescent (or only slightly so) in solid or colloidal condition. Stern showed that a suspension of living *Chlorella* has marked fluorescence. Finding that watery suspensions of chlorophyll are only weakly so, he added a lipoid, whereupon (after shaking) the fluorescence was strengthened. But the weakness of fluorescence in watery suspensions, as also in hard paraffin [Reinke, 1886] may be wholly due to the masking effect of dispersed light. Thus, the fluorescence of an alcoholic solution of chlorophyll may be wholly masked by shaking up a little

calcium carbonate in it, to return when the powder settles out. Liebaldt obtained similar results. As Stern observes, there must be an objective weakening of fluorescence since the materials which reflect the light reduce the stimulating as well as the fluorescent light by reflection and absorption. Similar differences in the intensity of fluorescence may be observed ultramicroscopically [Lloyd, 1923] in the living organism, but it may be argued nevertheless that the chlorophyll is in solid form, possibly adsorbed on protein; or further that even if dissolved in lipoid, the latter is colloidally dispersed as an emulsion, and the question remaining is the extent to which the interfaces of the various phases in the chloroplast may affect the degree of fluorescence. Stern has suggested that both these conditions may occur simultaneously in the same chloroplast, and indeed ultramicroscopically one may observe green particles and red fluorescence simultaneously. In some chloroplasts (*Vaucheria*) when suspended in glycerin, the pigment quickly collects in one to several minute vacuoles [Gaidukov, 1906], which can be seen to be individually fluorescent. This accords with Stern's inference. Furthermore, a pigment which has strong, deep red fluorescence but is pale olive green by transmitted light, occurs in the oil vacuoles of certain diatoms [Lloyd, 1923]. If this oil is the product of photosynthesis, as that in the chloroplast of *Vaucheria* is supposed to be [Meyer, 1918], and such oil takes up chlorophyll (or other oil soluble pigment in its vicinity), it may be argued that, if lipoid is present, the chlorophyll will be found in this by preferential solution.

It is unnecessary here to present in detail the various ideas which have been formulated in regard to the structure of the chloroplast, some of which at any rate have been conceived with no relation to the collophysical conditions which must subsist in all plasmatic structures [Strasburger, 1907; Priestley and Irving, 1907; Stern, 1921], and which also go beyond the hitherto available optical evidence. Assuming that lipoid is present, it may be predicted that the structure of the chloroplast will be found to be an oil-in-lyophile colloid type of emulsion. Iron, while not a chemical component of chlorophyll but necessary in catalysis, is considered by O. Warburg to be confined to restricted regions [Warburg, 1922] of the chloroplast. He found that a very small amount of HCN, which is adsorbed selectively by iron, suffices to stop assimilation. It is therefore to be considered that the iron may be confined to the lyophile phase, or even to certain surfaces, the interfaces between lipoid and water.

Pigments like lycopersicin [Duggar, 1913] are crystalline and therefore need not be considered here. What has been said concerning chlorophyll may be regarded as probably applicable in general to other oil-soluble pigments, at all events until we are possessed of more specific knowledge.

<div align="center">PROTOPLASM</div>

The conception of protoplasm as a colloidal complex needs no protagonist. The polemic begins when the nature of the complex is in question. If, in the beginning, the living substance had for its precurser a lyophile colloid, it is now a protein-carbohydrate-lipoid complex.

STRUCTURE OF PROTOPLASM

Any discussion of this material has to depart from an admission that not only does the structure of protoplasm in the "resting condition" of the

histologists, but also every living change, such as fibrillation, aggregation of materials into special organs (or bodies) such as chromosomes, etc., require elucidation. While we are far from this goal, measurable advances have been made, sufficient to give confidence that the point of view afforded by collophysics discloses matters not otherwise apprehended.

The attack on the question of structure dates from the classical studies of W. B. Hardy [1899], whose researches at a time when our knowledge of colloids was still young, led to the first real insight into protoplasmic structure. He represented a revulsive movement from a hypermorphological view of intracellular structure partaken in by Henneguy, Bütchli, and others.

Hardy showed that in heat irreversibly coagulable colloids (e.g., albumin) and in the gelatin type, the coagulated condition is external solid-internal water or the reverse, according to the amount of substance present. In the former case, the solid forms a network of granules which, e.g., in egg-white, offers a microscopic picture comparable to the structures so often described from fixed histological material, and which has become a sort of hieroglyph for cytoplasm in lieu of words.

The pertinent point in view of Hardy's work is indicated in the question as to the structure of an uncoagulated gel, admitting that, on coagulation, there are formed net-like structures which may undergo syneresis on contraction, releasing the alveolar water. For the biologist this question is directed to the structure of living protoplasm. The evidence must be obtained by the study of the properties of non-living gels and sols which offer analogies by such methods as used by Hardy and, more recently, by D. T. MacDougal [1921],[2] by microdissection [Barber, 1914, and others, W. Seifriz, 1921] and by microscopy, particularly with dark field illumination. The action of coagulants is not to be dismissed merely because it alters the microscopic appearance of a gel, since an understanding of what happens may lead to an understanding of prior structure.

The views regarding the structure of protoplasm derive largely from its behavior toward various solvents and solutes, and from the structure and behavior of emulsions and gels. It is difficult, if not impossible to discuss this phase of the matter without at the same time considering permeability. This is, however, a variable property which may depend in part but, we think, not wholly upon the condition of the surface of the plasmatic mass which is first affected by reagents, both decrease and increase occurring [Osterhout, 1922].

The momentary condition of the plasma (viscosity, permeability, etc.) may be due to the degree of dispersion of the internal phase or phases (Spaeth, 1916), the nature of these phases remaining in question. With regard to the theory of Free [1918] and Lloyd [1914], that the dominating phases are water-rich and water-poor lyophile colloids, Stiles [1923] has pointed out the objection arising out of the equal diffusion rates through hydrocolloids of different densities.

This idea, in more general form, can however embrace that of Lepeschkin [1911] which presents protoplasm as a complex of lipoids and proteins, dispersed in water in such fashion that the external phase is largely molecular water.

G. H. A. Clowes [1916] has postulated a non-aqueous internal phase, and, in order to explain changes in permeability, a reversal of phases, such as occurs in oil-water emulsions stabilized by soaps, though in protoplasm the

[2] "On the swelling of agar," see the critical studies of Fairbrother and Mastin, 1923.

stabilizers may be, partly, salts. The assumption that protoplasm is a liquid-liquid system is, however, unproved; and, while trying to avoid dogmatism, it now seems more likely that it is a solid-in-fluid system, with variable hydration capacities [Seifriz, 1923]. Nor does it seem likely that change in permeability depends upon phase-reversal [Seifriz, 1923 *]. It is indeed not going beyond the evidence to entertain a theory of the crystalline character of the solid constituent of the essential protoplasmic colloid complex, in which hydration changes involve changes in the size of the crystal-aggregates, a development of the view advanced by Sachs as early as 1865.[b] It is to Roentgen ray spectrography that we owe much strengthening of this conception, in the field of physics, though recently O. L. Sponsler [1922], using this method, has found difficulty in accepting it as regards starch grains. Nevertheless, in this connection, the idea of fluid crystals [Lehmann, 1906] may be fruitful, and indeed has, with considerable force, been found applicable by G. W. Scarth [1924] in explanation of colloidal changes associated with protoplasmic contraction. In an emulsion near the transition point of sol → gel, as protoplasm usually is in plants at least, very slight colloidal changes may produce great changes in viscosity. Thus a few degrees rise of temperature or a trivalent cation as dilute as one millionth normal may change the chloroplasts of *Spirogyra* from gel to sol with profound transformations of shape. These same bodies are an example of orderly arrangement of molecules in a colloid substance. In the gel state they rotate polarized light, while in the sol state this power is largely lost, but is regained on regelation. Evidently the colloidal micellae resemble fluid crystals which pack with a regular orientation. It is this structure which causes the chloroplasts to contract *longitudinally* with the first action of agents that lower viscosity, i.e., before the transference of water from internal to external phase has gone the length of separating the internal micellae.

This contraction is reversible and is a type of protoplasmic contraction in general, the mechanism of which can be solved only by due regard to the colloidal changes. It is pertinent here to recall the remarkable properties of certain protein crystals (such as the cubical ones which occur in the potato) which can be stained and swell with gradual loss of strict macrocrystalline form. We should know how the hydration has affected the internal arrangement of the crystal. Light on this might help us to understand protoplasm, as also might the further understanding of shrinkage phenomena [Liesegang, 1914] in gels.

The evidence afforded by ultramicroscopic examination leads to the conclusion that protoplasm, and even the chloroplast (in *Spirogyra*), may be optically void, but changes in this regard under various treatment are observable in the intensity of the Faraday-Tyndall effect. This, together with changes from iso- to anisotropism, observed by G. W. Scarth (l. c.) indicate hydration changes which may also involve variation in crystalline arrangement. Such changes in optical properties are certainly discernible, as, e.g., in the increased opacity of the plasma of sieve tubes, as compared with that of nearby living elements. Similar observations have been made by S. R. Price [1914], and by Ruth M. Addoms [1923]. This method has failed as yet to give support to the conception of the constitution of protoplasm as one of immiscible phases.

* See paper by W. Seifriz in this volume. *J. A.*
[a] "Physiology of Plants," p. 206-7.

We have not touched the question of organization. This involves direction of movement and correlation of emplacement of stuffs to afford the conditions for inheritance. A thoughtful discussion by C. M. Child [1920] may be referred to. Here we may only take space to iterate that the mobility and plasticity of the living body which consist of "organismic pattern" and the origin of this pattern itself, have been possible because of the scaffolding afforded by organic lyophile colloids, which can stabilize changes induced in response to environment. Of this, the fixation of polarity is a good example [Hurd, 1923].

PERMEABILITY

The property of protoplasm of permitting the diffusion of water and solutes, has been the subject of very numerous investigations, without, at the present moment, having brought us to a definite solution of the problem. It may, however, be said that by a process of elimination, a somewhat closer approach has been accomplished. The following theories have been advanced.

Ultrafilter or sieve theory. This dates historically from M. Traube's work, and has as its chief protagonist W. Ruhland [1914], who believes that the most complete analogy of behavior is that between the diffusion rates of dyes and their penetration through protoplasm. Ruhland's method of work and results have been most stimulating, and he did a service in committing himself to a clear-cut theory, even if it has seemed a somewhat crude one. If protoplasm were a rigid membrane like parchment, the view would have more appeal; but the flowing mobility of the living stuff, aside from other evidence, makes the conception difficult of visualization.

The *lipoid theory* appears in two forms. The earlier, formulated by E. Overton [1899] led him to the inference that the external layer of the protoplast is lipoidal, based upon the parallelism between lipoid solubility and penetration. The view breaks down most obviously in the light of the fact of the penetration of lipoid insoluble substances.

The form of the lipoid theory attributable to E. Nirenstein [1920] is based upon the parallelism between penetration of dyes and their relative solubility in a mixture of diamylamine and fatty acid, the former taking up acid, the latter basic dyes. According to Nirenstein this holds for *Paramoecium,* but Collander [1921] denies it in the case of plant cells.

Lipoid theories have gained wide notice, that of Mayer, and in particular of Overton, because as Winsterstein [1919] observes, it is a theory of narcosis in which the stress is laid on lipoid solvency. The mere fact that the narcotics are water soluble opened the whole theory to question.

Lepeschkin's [1911] studies, which betray great nicety of logic, indicate that the presence of lipoids, dispersed, along with proteins, in water as external phase, supply a complex whose behavior is to be explained only when the principle of partition in solution is taken into consideration. The effect of a lipoid solvent depends also upon its action on the protein and on its solubility in water.

The theory of J. Traube [1914, 1919] rests upon the surface activity of dissolved substances and states that their ability to permeate protoplasm is directly related to the degree to which they lower the surface tension of water. Here, also, Collander points out that in the basic (lipoid soluble)

and acid series of dyes, parallel differences in surface active behavior exist, but that, were the behavior at the surface between the various phases within the body of the protoplasm known, the theory might hold. But to this end these surfaces should be protoplasm-air surfaces.*

Closely related is the *adsorption theory* [Szuecs, 1910-13], according to which the passage of substances is determined by their adsorption by the plasmatic colloids. Assuming this to be purely an electrical phenomenon, as the effect of H-ions and those of polyvalent salts seem to indicate, this is another name for the *electrical theory*, which sees in the electric charge of the protoplasmic colloids the determining factor. That the dyes which do not permeate, or do so in very slight degree, have the electronegative character in common, is held by Collander to be decisive evidence for this conception, as is also their penetration when the H-ion concentration is high. This theory would allign itself most readily with the behavior of electrolytes.

Other factors than permeability probably affect the entry of solutes. The *vital staining theory* [Ruhland, 1912; Lepeschkin, 1911, p. 249; Bethe, 1916] states that all dyes except those whose colloidal particles are too large, can diffuse, but their visibility in the cell depends on the storage concentration consequent on adsorption by cell colloids, which again depends on H-ion concentration: in acid-content cells, acid dyes are adsorbed; in alkaline or neutral, basic dyes [Collander, 1921, p. 397]. R. Collander [1921] considers that this view applies with difficulty to plant cells, since their vacuoles are poor in colloids; but Robbins [1923] has found supporting evidence. Marian Irwin [1922] finds that the basic dye cresyl blue, penetrates and accumulates in the cell (*Nitella*) rapidly only when the external medium has a pH higher than that of the sap (pH 5.6). It is apparent that a profitable field of research would be found in studying the penetration of dyes into cells containing such colloids, since the adsorption of dyes by these [Lloyd, 1920] would serve as a criterion of diffusion through the enveloping protoplasmic membrane. It is obvious that there may be dyes which can be so adsorbed but which cannot penetrate the protoplasm, and their adsorption would be then hindered. Adsorption of dyes by the protoplasm itself in the living condition, or, to put it otherwise, vital staining in an exact sense, is extremely doubtful. Of the series of dyes studied by Collander, not one acted thus (*loc. cit.*, p. 375) and the writer found that, although neutral red penetrates the protoplasm of the parenchyma cells of *Opuntia Blakeana* and other species easily and rapidly, and the tissues can be kept alive for a number of days in water, the moribund condition of the protoplasm is at once indicated by the fact that the dye then, but then only, begins to be adsorbed by it.

It is obviously impossible to discuss the above theories within a short space. It may, however, be remarked that more recently there has occurred a broadening of method which appears to be bringing quantitative results of significance. The point of view which presents protoplasm as a mixture of ampholytes bathed by a buffer solution [Haynes, 1921] seems to be pregnant with promise. The correlation of changes in hydration, viscosity, optical properties, etc., with changes in H-ion concentration in gelatin and other emulsoid colloids with those in protoplasm, proceed from this point of departure [J. Loeb, Osterhout, Fenn, Scarth, etc.].⁴

* See paper by J. Traube in Vol. I of this series. *J. A.*
⁴ For the literature see W. Stiles, "New Phytol., 1921.

VISCOSITY

Viscosity as a property of protoplasm has been recognized since the time of von Mohl, and has been the subject of much interesting and ingenious experimentation and observation. Von Mohl himself observed that the viscosity increases with age. Viscosity and permeability probably do not run parallel [Osterhout, 1916], and the non-miscibility of protoplasm with water, when this obtains, cannot be held to be due merely to viscosity [Seifriz, 1921] though when protoplasm does mix with water, it is in a state of extreme liquidity.

The range of viscosity values of protoplasm is doubtless very great. Seifriz has attempted to formulate standards usable in microdissection studies, and this is a step in the right direction. He finds that reversible solation-gelation occurs during the formation of pseudopodia. The surface layer shows remarkable increase in rigidity on being drawn out by a fine needle point. The surface layers of *Spirogyra* protoplasm under various conditions of plasmolysis betray similar changes [Weber, 1921]. I [1924] have observed such under chloroform narcosis. On the other hand, Doflein distinguished in the pseudopodia of the Rhizopods an axial stereoplasm and a more fluid ensheathing rheoplasm, a structure which permits a more flexile structure. It may be that the movements of diatoms are connected with a similar structure. (See Seifriz, *loc. cit.* Also his paper in this volume.)

Local intracellular differences in viscosity occur during mitosis [Nemec, Spek, 1920] and these indicate that changes from sol to gel are of paramount importance during the complicated steps involved.

The methods of evaluation of viscosity in plant cells at present available are as follows:

1. Rate of movement of solid inclusions upon change in position of the cell or tissue, e.g., of starch grains, or, when possible, of introduced particles (iron particles in the plasmodia of *Myxomycetes* moving under the influence of the electromagnet [Heilbronn, 1918]. Measurements have been made with the horizontal microscope on the rate of fall of starch grains in the starch sheath of *Phascolus* [Heilbronn, 1914-1918].

2. Rate of change of form or position under centrifuging (Weber discovered positive and negative changes in viscosity under the influence of narcotics by centrifuging) or otherwise, of cell organs, e.g., the chloroplasts of *Spirogyra*. Changes in optical appearances referable to sol-gel interchange and changes from isotropy to anistropy between crossed Nichols, have been observed in this plant by means of dark field illumination by G. W. Scarth.[*]

3. Changes in rate and amplitude of (Brownian) movement of particles [Bayliss, 1920]. V. Henri [1908] used the cinematographic method with conspicuous success in the study of Brownian movement of caoutchouc suspensoids in latex, and the effect of various reagents on this.

4. Resistance to physical manipulation, e.g., crushing, plasmolysis and especially, microdissection. The last, introduced by Barber and developed by Chambers, Kite and Seifriz, has yielded very useful results amplifying by means of careful and exact manipulation, what had previously been attempted by the grosser and more accidental methods. Nevertheless resistance to changes of form imposed by plasmolysis which has the advantage of not

[*] See also paper by L. V. Heilbronn, this volume. *J. A.*

injuring the cell, is, in some cases [e.g., chloroplasts of spirogyra, Scarth, 1923] a useful test.*

Knowledge of change in viscosity in various non-living lyophile colloids under various treatments, affords analogies which, as for swelling, and other properties, enable us the better to judge of the behavior of protoplasm in this regard. The behavior of gelatin and of albumin on heating, for example, leaves little doubt that the latter furnishes the closer analog, as also its behavior toward acids in higher concentrations. As an instance in point may be mentioned the coagulability of protoplasms (of pollen) by acid [Lloyd, 1915].

CELL SAP

The earliest study of the viscosity of the cell sap was made by Ewart in 1903, followed by Heilbronn in 1914. Still later Weber [1921] arrived at a temperature coefficient of 1.13 to 1.19 for each 10 degrees difference of temperature between 40 degrees and 0, the limits of experimentation. Such changes vary much more widely in relation to temperature, acidity, etc., when colloids are present, than if only crystalloids are present. Hence the method employed may lead to knowledge of the intimate physiology of the cell, as e.g., the rate of tropistic responses in which also changes in acidity may play a related rôle [Small, 1920; Newcombe, 1923].

Cell sap frequently, if not indeed always, contains lyophile colloid which, in some instances, becomes highly concentrated and even jelled, so that the viscosity increases enormously [Lloyd, 1911]. The mucilage cells which secrete calcium oxalate raphides, have always highly viscous sap. (For further remarks see beyond, under Latex.)

GROWTH

Growth presents two general aspects for consideration: (1) increase in volume, which may occur with or without a net increase in dry weight, and (2) acquisition and fixation of form.

Increase in volume involves osmotic pressure and imbibition. The former can obtain because the colloids of the body, in particular the plasmatic membrane or mass, have the property of semipermeability. Plant cells, from this point of view, have been regarded as closed systems, inclosing solutions of higher osmotic pressure than the solutions of the adjacent environment, this pressure serving to distend the cell membrane, including the cell wall, thus furnishing the condition for a step forward in increase in size. The discovery in 1867 of non-living membranes having the property in question toward crystalloids by M. Traube [5] furnished the starting point for a remarkable epoch of investigation. These membranes are insoluble tannates or hydrates, and are of a colloidal character.[6] When suitable conditions are supplied the membranes will "grow" and acquire external forms which stimulate to a degree the forms of plants. Stéphane Leduc [1910] has furnished notable examples of these.* Incidentally to the study of the tannin in fruits, it was found that tannin which diffuses from the tannin cells, forms precipi-

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* See paper by R. Chambers in this volume. *J. A.*
[5] See Livingston, B. E., "Rôle of diffusion and osmotic pressure," and W. Stiles, 1923.
[6] J. E. F. af Klercker made and studied artificial cells of microscopic dimensions containing tannin by means of which he established similarities of behavior of tannin in them and in cells. (1888).
* See paper by S Leduc, this volume. *J. A.*

tation membranes with great variety of form if the pulp is allowed to stand. These membranes remain unaffected for many years, probably indefinitely [Lloyd, 1911, 1922].[7]   J. Rosett [1917] devised a method for obtaining very extensive membranes with potassium permanganate, forming membranes in a mixture of sodium silicate and sodium salicylate.   More recently Ralph S. Lillie and Earl N. Johnston have produced growing precipitation membranes resulting from local electrolysis at metallic surfaces.   Lillie has given in his discussion an acute analysis of the comparisons between the growth of such non-living systems and that of living organisms and with more than usual respect for the devil's advocate [Lillie, 1917, 1919, 1922].   The building up of external or more or less superficial deposits of salts in, and conditioned by, surrounding mucilages, and in more compact colloidal walls by the Cyanophyceae and other algae (simple relatively as these are from the physico-chemical point of view, and involving such problems as the origin of tufas [Jones, 1914]), results oftentimes in forms very similar to the precipitation membranes above mentioned; but their origin is to be understood rather in the light of the effect of colloids on crystallization, the total form being conditioned by that of the pre-existing organism.[*]

Precipitation membranes having diosmotic properties may perhaps also be crystalloidal.   When crystals of calcium carbonate are exposed to oxalic acid or to ammonium oxalate and acetic acid, precipitation membranes, which are apparently entirely crystalloidal, are formed on their surfaces, and grow at rates and to an extent permitted by the conditions (concentration of acid, amount available in relation to the calcium present).

All such membranes are, however, exceedingly simple as compared to cell membranes which are complex mixtures of lyophile proteins, carbohydrates, lipoids and soaps of these.   The artificial cell protected by them can, therefore, afford only a faintly limned picture of what obtains in the living cell.   Models which approach somewhat more closely to the latter have been devised by Martin H. Fischer and by D. T. MacDougal.   Fischer made cells of soap (sodium stearate) whose membrane may be changed as to its concentration, resulting in different filtration capacities, that permit filtration according to the amount of free water in the cell contents (accompanied by changes from acidity to alkalinity), displays change of permeability in the presence of salts, and furnishes an analog to the effect of distilled water [Fischer and McLaughlin, 1920].   MacDougal [1922] in recognition of the importance of lipoids, has devised models of the cell by variously lining porous thimbles of terra cotta with agar, gelatin, lecithin and various combinations of these, finding behaviors in terms of positive and negative osmosis varying with hydration effects upon the imitation plasmatic colloids.   Crude as these contrivances admittedly are, they serve to assist us to a comprehension of the plasmatic mechanism.   Their very imperfections point by negative signs to better roads towards perfection.[8]

It is now, however, certain that imbibition plays an important rôle in growth.   Just as the hydratation distention of the vacuolated cell may express the osmotic pressure of the solution of electrolytes and non-electrolytes inclosed within the vacuole, precisely similar pressures may be exerted in the same type of cell by inclosed colloids to an extent indeed sufficient to burst the

[7] Or see Dekker, "Die Gerbstoffe."
[*] See references to W. M. Ord in index to this volume. *J. A.*
[8] An analogy to the cell: E. N. Harvey: "A simple method of making artificial cells," etc., *Science, n.s.,* **36,** 564-5 (1912).

cell[9] [Lloyd, 1911, 1922]. That the distensive forces of meristemmatic cells, before vacuoles have been formed (e.g., many pollen grains), are also referable to the imbibition capacity of the protoplasm, seems obvious [Kunkel, 1912; Antony and Harland, 1920], but evidence has been furnished which gives force to the idea. It requires amplification, to be sure, and the experimentation is full of difficulties of interpretation. The starting point is furnished by the studies of swelling of colloids such as gelatin, agar, fibrin and the like. MacDougal, in the botanical field, has busied himself extensively with the hydration capacities of various mixtures of colloids and of plant tissues. The results are considered elsewhere in the present volume, as also the subject in general.

Here we confine ourselves to *ad hoc* experiments on growth of cells or tissues in relation to the acid or basic character of the milieu. G. A. Borowikow [1913] proceeded from the observations on the effect of acids and alkalis on emulsion colloids, and the contrary effect of salts, and studied the effects of various agents on the growth of the elongation-zone of the root of seedlings of *Helianthus annuus*. Those agents which increase the "hydration of colloids," namely acids and alkalis, were found to increase growth rates, with the exception of alkalis which formed salts with the CO_2 set free by the plant respiration of the seedlings. Growth, therefore, is the result of hydration of the plasma. In another paper Borowikow [1914] afforded data to show that high growth rates are not related to high concentration of the cell sap as generally supposed, but the reverse rather. The important period of growth is that during which the meristemmatic cells enter upon the stretching phase, and is characterized by a change of the plasma and possibly of the cell-wall from gel to sol condition. This hydration brings them into condition for extension by turgor. Osmotic pressure is therefore secondary in its rôle [Mazé, 1916; Dachnowski, 1914; Long, 1915; Maquenne and Demoussey, 1918; Reed, 1921; Hoagland, 1917].

In order to eliminate the factor of osmotic pressure, Lloyd made use of embryonic cells with no vacuoles (pollen cells of *Phaseolus odoratus*). These cells imbibe water so vigorously that they burst. This may be controlled by cane sugar in 20 per cent or higher concentration. By combining sugar with acids and alkalis it was found that the maximum growth rates occurred at about N/3200, and that the general behavior at various concentrations of the reagents is analogous with the swelling of gelatin [Lloyd, 1917, 1918]. It is to be noted, however, that the concentrations of reagents which cause increased growth are very much lower than those which are required for the swelling of gelatin, but which are lethal in their effect upon protoplasm.

The optically observable effects of the H-ion on the protoplasm of root hairs have been studied ultramicroscopically by Miss Addoms [1923]. Distinct flocculation effects could be traced within a range of pH 3.94 and 3.47, these concentrations being much higher than those at which increased growth rates have been observed, and are due to dehydration [Duggar, 1910; Wolf, 1921; Tarr and Noble, 1922].[10]

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[9] O. Loew (1906) long ago recognized the "water binding capacity" of the colloids of the vacuole.
[10] Growth promoting and inhibiting substances ("Hormones") are not considered here, since they have not been objectivated as yet. (Reed, 1921; Tschirch, 1921; Haberlandt, 1921; Wright, 1922; Appleman, 1918.)
Tumour tissue; oedematous tissues. Erwin F. Smith (1917) found that overgrowths are readily produced by chemical stimulation, suggesting this to be the result of local osmotic action. R. B. Harvey (1920) found a slightly lower H-ion concentration in tumours as compared with healthy

SAP

Sap accumulates in vacuoles which in mature parenchyma cells may occupy much the greater part of the cell volume. Aside from the various solutes (acids, salts, sugars, pigments, etc.), oils and resins, it contains also proteins, enzymes, mucilages, tannins, inulin, etc., in colloidal condition, more rarely caoutchouc (*Parthenium*), etc. Of the proteins, Loew and Bokorny [1887] have attached much importance to "labile albumin," but the existence of this substance has been vigorously contested by C. van Wisselingh [1914], who regards the reactions upon which these authors based their views as those of tannin-alkaloid. Ultramicroscopically, however, one observes minute suspensoids [Price, 1914] in most if not all cells, and these are probably in part protein, but sometimes they are merely minute crystals (calcium oxalate, etc.). I have been unable to substantiate W. Flemming's [1882] statement that osmic acid produces a reticular precipitate in the sap of *Spirogyra*, in which van Wisselingh [1914] believes no proteins occur.

Mucilages (carbohydrates) occur very generally, but are difficult of identification except when in considerable concentration. They, in common with external mucilages, etc. [limonite, Steinecke, 1923], undoubtedly play a rôle in determining the character of crystallization, as in raphide cells, in which acicular calcium oxalate crystals occur in fascia. The other forms of calcium oxalate (single, double, "druses") can form without the presence of colloid, and it would therefore seem that with respect to these, colloids in the cells are of minor influence [Jeffrey, 1922; Lloyd, 1923].

Recent experiments done by myself have shown that, for sulfur, methylene-blue-KNO_3, cane sugar and some of the commoner salts, it requires indeed a very high viscosity of the medium in which crystallization is occurring to procure an appreciable effect upon the form of the crystals, or crystalline body, obtained, or upon the rate of crystallization; but when the viscosity is sufficiently high, very marked, if not profound changes may ensue. The direction of such changes is, with increasing viscosity, toward a slower rate of crystallization and toward the formation of smaller crystals until, indeed, spherocrystalline bodies are produced, whose structure is no longer coarse enough to yield to microscopic resolution. These bodies show particularities which ally them to starch, inulin, etc., in the spherocrystalline state. It must be said in this connection, however, that many of the crystalline forms observed by others, and regarded as due to the presence of colloid, are in no wise different from those obtainable without the presence of colloid. Rate of crystallization depends, among other conditions, upon the rate of diffusion of the material through the colloid, and it is known that for noncolloids this rate is not affected until a very high viscosity is reached. If, however, a low viscosity accompanied by a very high rate of crystallization occurs, again very small crystals in a feathery body can be obtained, as when a thin film of a metastable solution is evaporated, e.g., sulfur out of xyol. Here the possible rate of diffusion through very thin films is a limiting factor.*

Carbohydrate bodies [Clark, 1913] may act as adsorbers of tannin limiting its freedom. Tannin when thus "protected" cannot be precipitated by alkaloids or other color-producing reagents. With the latter, the color only

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* The work of W. M. Ord and those of his time is most remarkable, but has been overlooked. See "The Influence of Colloids on Crystalline Form and Cohesion," London, 1879. Also J. Alexander, *Kolloid Zeitschrift*, 1909. *J. A.*

appears, and the tannin is not detectable as astringent. This suggests that lyophile colloids may have a wide and various function in protecting materials harmful to protoplasm, and so preventing their toxic effect.[11]

Even crystalloids may act thus, e.g., sugar [Knudson, 1913]. Many relations would become intelligible on the application of this principle.

Caoutchouc may collect in the sap vacuoles of plant cells to form masses. Its accumulation here appears to arise from the syndrome of numerous minute suspensoids arising freely in the protoplasm [Lloyd, 1911; Hall and Goodspeed, 1919]. A somewhat similar procedure takes place in a latex plant, *Musa*, [Lloyd, 1920], under certain conditions. Concerning the genesis and secretion and behavior of this and similar substances (oils, resins, mucilages) we have only the vaguest ideas. (See glandular action, *infra*.)

The condition of dispersion of colloids in the cell-sap doubtless is connected with the acidity. Inasmuch as sap expressed from tissues is always accompanied by protoplasm in changed condition and by a variety of materials disturbed in their mutual relations, the determination of the H-ion concentration of juices thus derived is not to be depended upon as indicating that of the sap itself *in situ*, whether or not proteins are present in the sap. Change in acidity may be indicated by indicators normally present (as when petals change in color) in the absence of which introduced indicators may be used. The sap of marine plants is almost neutral, while that of land plants shows a great variation pH 1.4 to 8 (but is rarely alkaline), according to the species and to circumstances [Atkins, 1922]. Edith P. Smith observed that the pH of the cell sap of the corolla of *Ipomaea* changed from 7.8 to 6 as the concentration of CO_2 in the water increased [Smith, 1923]. Further the sap in common with the liquid phase (water-solvent) of the protoplasm exerts a buffer action [Haynes, 1921]. This important action of cell physiology has been little investigated.

LATEX

Between sap and latex we may draw only an arbitrary line of distinction, most conveniently determined by the occurrence of the latter in vessels, though this criterion breaks down on attempting a definition of "vessel." Latex, therefore, is the body fluid found in vessels, and which oozes from them on injury, usually under very considerable internal pressure. As this pressure varies during the day, the rate of exudation of latex (*Hevea*) is highest in the night and early morning, going down as transpiration increases.[12] It is a common mistake in speaking of latex to think only of rubber latex or that containing balata or gutta percha. The commercial importance of rubber during its period of apotheosis, scarcely drawn to a close, has narrowed the meaning of the term in general practice. We need think, as a corrective, only of the latex of the poppy, which contains no rubber, but the alkaloid morphine, or its chemical antecedent [True and Stockberger, 1916], a substance which seems [Annett, 1920], in the light of present evidence, as useless to the plant as rubber [Bobilioff, 1915], appears to be. Naturally, commercially important latices are best known as to their composition.

We may here, however, only review the more important characteristics

[11] Lepeschkin's results permit the theory that the lipoid-rich internal phase came by taking up narcotics prevent their accumulation in the plasmatic albuminoids.
[12] For detailed description of latex from various sources, reference may be had to H. Molisch's book "Milch- und Schleimsafte in Pflanzen" (1901), and to that of Zimmerman, "Der Manihot Kautschuk" (1913).

of these fluids, which are emulsions of hydrophile colloids, or of hydrophile and hydrophobe intermixed. The latter are conspicuously caoutchouc and kindred substances, some resins, fats. These occur as suspensoids ranging in size from 0.1 micron or less in *Kickxia* [Spence, 1909], to 50 or more microns in *Musa* [Trecul, 1867; Lloyd, 1920]. As is evident from ultramicroscopic examination of *Musa* latex, the caoutchouc suspensoids are composed of more than one stuff, since they betray a discontinuous structure. I have isolated from them at least two albanes, which appear to be associated in the globules. The minuteness of most suspensoids prevents observing this distinction, which, however, probably occurs, as is evident from the fact that the caoutchouc suspensoids of *Hevea* are not always spherical [Bobilioff, 1919], as I have myself observed. Pear-shaped globules are frequent.[13]

The hydrophile colloids occur either in solution (or amicronic suspension) or in suspension, as colorless spheres of all sizes within the ordinary ultramicroscopic limits. Pigments also occur (e.g., Papaveraceae, Euphorbia, etc.). All such suspensoids have lively Brownian movement. The amount of such present may be so small, or the dispersion may be such, that the latex appears quite limpid macroscopically, betraying its character only through a Faraday-Tyndall effect when a strong beam of light is used, but upon ultramicroscopic examination is seen to contain suspensoids of minute size and of probably entirely hydrophile character (e.g., *Cucurbitaceae, Ceropegia dichotoma*).

2. *In vivo*, latices are stable, and in this respect as in their behavior upon wounding, may be compared with the blood of the animal body. They vary, however, in their power of spontaneous coagulation, which occurs quickly at the cut edges of a wound (leaf of *Ficus elastica, Cucurbitaceae*) or more slowly (several hours being required) at the cut ends of the latex vessels (*Hevea*) or not at all. That of *Ceropegia* may be dried and resuspended. That of *Alocasia sp.* forms in osmic acid vapor, at the air-water surface, a concentration film of a lyophile colloid which solidifies. Coagulation occurs *in situ* under pathological conditions, as during the formation of lesions in *Hevea* due to the obscure disease called Brown Bast, and in the latex vessels of *Musa,* resulting in the plugging of them by the caoutchouc coagulum of each constituent cell of the vessel, a condition which is brought about by "chilling" in transport, as it is called in the trade [Lloyd, 1920]. With the ageing of laticiferous tissues and the formations of bark, or as symptomatic of disease, e.g., Brown Bast, the latex may coagulate (*Hevea*). The stability of latex is due, according to V. Henri, to the electric charge on the suspensoids. Latex (*Hevea*) is a negative emulsion, the caoutchouc suspensoids moving, in dialyzed latex, to the anode. Coagulation by electrolytes (acids and salts of bi- and tri-valent metals) is determined by positive ions [Henry, 1907]. The charge can, however, not be the only factor. Surface active hydrophile colloids (possibly resin soaps, soluble proteins [14]) are certainly present, and would, therefore, contribute to the stability of the emulsion [Henri, 1908; Whitby and Dolid, 1923]. According to G. Vernet [1919] [15] coagulation results on rendering the proteins insoluble, or when these ampholytes are coagulated [Lindet], or flocculated, e.g., by freezing, as in the case of milk. My own ultramicroscopic observations lead me to favor this view. The H-ion

[13] Loomis & Stump (1923) state this to be always the case in *Hevea* latex preserved with 5 per cent ammonia, but I think this questionable.
[14] Attempts to make artificial latices by redispersing the caoutchouc proceed on such an assumption (Davis, 1923; Stevens, 1923).
[15] For other views see Zimmerman, 1913.

concentration of the latex of *Hevea* is very little on the acid side of neutral, or neutral [Bobilioff, 1919]. The behavior of the proteins toward acids (which cause coagulation) and alkalis (which cause increased stabilization [16]) agrees with this idea. The less effective rôle of formaldehyde as a stabilizer is not so well understood.[17]

The behavior of banana (*Musa*) latex is just the reverse of that of *Hevea*, in that it is stabilized by acid and coagulated by alkali. When treated with ammonia coagulation sets in with great rapidity, and the rubber suspensoids, without any alteration or syndromy, become firmly encased in a translucent coagulum. Since tannic acid occurs in this latex, it is a permissible theory that this acid is its natural stabilizing agent. This behavior comports with the idea of Vernet, above cited.

The coagulation of some other latices from which caoutchouc is absent consists in the irreversible flocculation and gel-formation of hydrophile colloid, the ultramicroscopically visible suspensoids becoming the internal phase, the external gel being optically empty (*Cucurbitaceae*). Quite similarly the caoutchouc suspensoids on complete coagulation compose an *optically empty continuum*, the external phase, containing disparate protein (and doubtless other) suspensoids (*Hevea, Ficus*). There is no fibril or net-like structure, which may appear, however, but only incidentally to incomplete syndromy of the caoutchouc globules. The ease with which this takes place depends, among other things, upon the size of the suspensoids, the largest suspensoids displaying syndromy with great readiness, as I have observed in *Musa*, the size and viscosity of the globules of which permit them to spread upon an air-water surface, forming a greasy looking pellicle. Henri (*loc. cit.*) observed that coagulation of *Hevea* latex takes place at the top of a column of latex, where, due to creaming, the largest globules occur.

To what extent enzymes take part, and even whether they are present or not, is uncertain. On general grounds, and the known occurrence in a number of cases, as in the sap of wood [Wotchal, 1916], we are led to expect them. It may be mentioned that the sap of some plants is used to procure coagulation of the latex of others, but of the precise mechanism involved we know nothing.

THE INTERNAL STRUCTURE OF THE MULTICELLULAR PLANT BODY

In regard of internal structure there are two widely diverse forms of plants, the syncytial, and the cellular. The syncytial forms, though now relatively inconspicuous in point of numbers and size, and are confined to a water environment, may have attained great size in early geological times.[18] The conspicuous feature in these plants is the continuity of protoplasmic structure, which in multicellular forms appears to be more or less discontinuous. This condition, which is probably more apparent than real [Noll, 1903], arises from the development of cell walls, which in plants are certainly more conspicuous than in animal tissues.[19] Historically the cell wall has in large measure dominated observation and reasoning, as witness the word-fossils

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[16] Rubber latex stabilized with ammonia is now transported in fluid condition from Sumatra to New York.

[17] It is pointed out to me by Dr. A. E. Jury that formaldehyde may form a compound with proteins present, or merely coagulate them. Latex treated with formaldehyde has a higher viscosity than ammonia latex, and appears to some extent to be unstabilized. But in destroying bacteria it prevents the accumulation of acids.

[18] *Nematophyton*, a huge Devonian alga, seems to have been of this type.

[19] An interesting view, but of doubtful application to the plant has been advanced by L. Loeb (1922).

"cell" and "tissue." The cell is still the "unit of structure," and the conception in more or less vague form, that cells are like bricks in a wall, still rules our ideas of tissues. Dissent from this conception may be traced in the controversy over the question of the interrelation of form and cell structure [20] in the studies of protoplasmic continuity [Strasburger, 1901] of intercellular protoplasm leading to negative results [Kny, 1903, 1904], and in the attempts to localize the "formative stuffs" and to explain the correlation of structure and growth, and the transmission of stimuli. The presence of mechanism of transport to some degree has been taken as alleviating the difficulty, for it is tacitly or otherwise assumed that movements of growth promoting or inhibiting substances [Sachs, 1887] are thus effected. The transmission of stimuli across dead regions [Arthur and MacDougal, 1898]; and through glass tubes [Ricca, 1916], and after removal of the cortex, seems to point to the vascular regions (wood, *in Mimosa*) as those of transmission, but we also know that such stimuli are transmitted in the living parenchyma after the removal of the vascular tract [Oliver, 1887; Lloyd, 1911; Newcombe, 1922]. But more than this, R. Snow [1923] has shown that in the root tip of *Vicia faba*, the excitation can be transmitted across an intercalated zone of gelatin, between the decapitated root apex and the stock [Stark, 1921; Stark and Drechsel, 1922]. Ricca believes the agent of transmission to be a "hormone." There must be such transmission at all events over narrow zones of tissue, at least in motile organs such as the pulvini of *Mimosa pudica*, to cite only one obvious instance. It is difficult to find an adequate explanation of the physiological concatenation, and it has been sought in the protoplasmic continuities which Strasburger called "plasmodesmen," a word which seems to have resisted a happy transposition into our own tongue. The fact that these structures occur between the cells derived from different parents in graft hybrids, and between parasite and host, seems to indicate that their origin is not, as previously thought, connected with spindle "fibers," but is secondary, on the assumption that the naked protoplasts are "never immediately contiguous." [21] In making this remark, Miss Hume indicated the fundamental question [Hume, 1913] to which we have at present no answer. The assumption is, however, certainly open to inquiry. Some very suggestive work has been afforded by Hansteen-Cranner [1914, 1919]. The presence of fatty acids, phosphoric acid, glycerol and nitrogen in the cell-wall substance proper establishes, this investigator holds, the presence of lipoids in the cell membrane. The cell wall, he believes, is not cellulose, but some sort of a mixture of this and pectin together with water- and alcohol-soluble lipoids, constituted a colloidal complex which is really the outer layer of the living protoplast, physically a concentration layer inclosing the protein phases of the protoplast. Incomplete though it may be at the moment, the picture thus furnished is one in which the continuity of the living body dominates the structural elements, and the cell-wall may be conceived as lying in the living substance and not merely bounding it. How far this conception may receive substantiation remains to be seen, but there are behaviors which contribute support to the view. Thus the excretion of water-insolubles appears less difficult to understand. Thus also the question of the adhesion of the protoplasts to the cell wall observed upon plasmolysis by strong solutions, and after various treatments which have been thought to affect the surface of the protoplasm, would

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[20] See E. B. Wilson, "The Cell," in "Development and Inheritance." Also his paper in this volume.
[21] For the literature see Sharp: Introduction to Cytology.

take on a different aspect. The persistence of the hundreds of minute cytoplasmic threads under certain treatments observable in *Allium* epidermis and in *Spirogyra* [Weber, *loc. cit.*], especially from the septa, may be regarded as evidence that the substance of the layer of cytoplasm near the cell wall is less easily *broken* than otherwise, rather than separated from a wall against which it is supposed to lie. When translated into other terms more acceptable to the physicist, this may be a lowered viscosity, or change from gel to sol, or decoagulation, but not into more or less adhesiveness to the cell wall [Scarth, 1923]. Whereas, however, "breakage" at the inner margin of the cell wall by the same agency is a harmless and reversible accident of ordinary plasmolysis, rupture of a deeper layer is immediately fatal [Scarth, *loc. cit.*]

Of more than usual interest here is the compound prepared from pectin and lauric acid by Hansteen-Cranner [*loc. cit.*] which presents certain rather close analogies to the cell wall. From his experience grew the suggestion that soaps may be present and to this may be attributed the properties of the wall membrane, which normally presents marked adsorption capacities, demonstrated by Devaux [1901].

Briefly, and without minimizing the difficulties, the work of Hansteen-Cranner furnishes a point of view from which one regards the delimitation of the protoplast at the inner surface of the cell wall as non-existent, but that there is in reality a gradient of protoplasm toward a minimum at the outer face of the cell wall, and gradients of lipoids, and of pectin in the reverse sense. That this gradient is a precipitate one at the face of the cellulose may be admitted. Hansteen-Cranner found no protein to diffuse from the cell wall, so that it cannot be present in the surface layer. The phase relation of water-soluble and water-insoluble phosphatides and their mutability in this regard is considered to be responsible for changes in permeability, and thus a semipermeable layer may after all be disposed of [Fischer, 1902]. Whether this is the key which is to open this biological Pandora's box or not, it seems logically necessary to think more about the cell wall than hitherto, as an integral part of the living protoplast. The cellulose or its precursor (pectin) is now a secretion within, but at and near the surface of the protoplast, the protoplasm gradient and that of the surface active substance displaying inverse ratios. This view, which consists with what is known of the manner of growth in the usual sense, has been formulated by W. Robinson [1920], who believes that the microscopic effects of the various forms of stress in cell walls do not wholly support Nägeli's micellar hypothesis, though it is quite possible to show that this may be brought into harmony with the particular form of the theory advocated by Robinson [Scarth, 1924]. In employing surface activity to account for the origin of cell walls, the greatest difficulty perhaps is met with in explaining that of the primary cell wall, commonly spoken of as the "cementing substance," an expression permissible in a figurative sense, but having no significance with regard to origin.

In relation to the above the mobile nature of young tissues may be recalled. Tissues, even such as become sclerenchymatous, are remarkably fluid during their development, growth in diameter and length, especially involving necessary and extensive surface-to-surface readjustments of contiguous cells, as, e.g., in the phloem sclerenchyma of *Parthenium argentatum* [Lloyd, 1911; Renner, 1910]. These readjustments, a beautiful example of which is supplied by the developing druse cells in *Myriophyllum,* are really of very common occurrence. It is evident that they are constantly occurring during the

development of cortical parenchyma, where mitotic division produces tangential series of cells. Inasmuch as the mitoses of radially adjacent series are not simultaneous, there must result a natural adjustment by mutual slipping. The mode of growth of medullary ray cells between the tracheids in the Gymnosperms further exemplifies the same condition.

This fluid condition of tissues can result also in the development of intercellular spaces. Under pathological conditions the middle lamella is attacked by enzymes, even in advance of the offending organism, and analogous changes occur in the root cap, and during adscission by which the cells of the tissues are mutually freed. [Lloyd, 1920; Sampson, 1918, and others.] But the development of intercellular spaces seems to involve other processes such as autolysis, sliding, &c. When such spaces have been established, changes then occur at the air-cell wall surface (if such be established), the rate and extent of which depend upon vapor pressure and, probably, also, upon the tensions of the various gases present.

It is obvious that the stratification of the environment into a water-solid complex, the soil and a water-vapor-air complex, the atmosphere furnishes an antithesis in respect of conditions imposed at the surface of the organism. The implications are too many and multifarious to permit even a cursory survey here. We must therefore limit the discussion to very general considerations.

Submerged plants in general, more especially those with an ancestry always subjected to the same habitat, have external walls of mucilaginous or gelatinous character (Algae). The portions of land plants exposed to air are modified externally with waxy material so as to be lyophobe [Blackwell, 1913], while their underground parts, although related to water-adsorption and may be wetted [Rosenthaler and Kolle, 1921], show suberization, cutinization and other local peculiarities of permeability which, on general grounds, may be considered rather unexpected; as, e.g., the resistance of the root cap cells in *Vicia* to the passage of water, due to the still unknown composition of the cell-walls [Priestley and Tupper, 1922] which according to Priestley [1920] have the property of semipermeability toward certain dyes similar to that of protoplasm [Priestley, 1920].

Lyophobe membranes occur, however, within the organism [e.g., Casparian strip, Priestley and North, 1922], notably in seed coats, rendering them semipermeable, or even permeable in one direction and not in the other [Denny, 1917]. This matter is one which deserves much more study [Rippel, 1918]. Some internal lyophile cellulose walls are more capable of taking up water than others, as, for instance, those of amyloid [giving a blue iodine adsorption reaction, Blackwell, 1913], occurring, e.g., in the cacti [Lloyd, 1919] and in the date endosperm during development [Lloyd, 1910; Ziegenspeck, 1919]. Amyloid walls are much more distensible than those composed of ordinary cellulose, and their optical properties under strains may be expected to be similar to those of tendons [Quagliariello, 1922; Correns, 1891].

Structurally, the cell wall is indeed probably micellar [Zimmermann, 1884] and its growth by accretion [van Wisselingh, 1912], and may be accompanied by breakage of the outer wall to accommodate the newer inner strata, as I have observed in growing pollen tubes of *Phaseolus*. During growth, the outer strata may be gelatinized, as in abscission cells [Lloyd, 1916], and among the algae; or the inner strata may suffer similar changes [Lloyd, 1919] as in the case of the mucilage cells of the cacti, as to the precise origin of the mucilage,

in which, however, there is difference of opinion [Stewart, 1919]. The relation of structure to imbibitional properties is of greatest importance practically as well as theoretically, and this needs careful examination. v. Hoehnel [1884] found that wood fibers during swelling at first lengthen and then shorten. The grosser behavior is well known.

The cell wall shows considerable adsorptive activity, for metals [Hoyt, 1914; Devaux, 1901] but not for salts, according to Scheringa [1920] and Kostychev and Eliasberg [1920]. But Emstander [1920] regards the destruction of certain plants as the result of adsorption of Cu-salts from a rain water solution in the neighborhood of telegraph wires. The walls of the water vascular system may show adsorption of salts according to Flood [1919].

The accumulation in heart wood, of secondary substances, to which it owes its properties, often of resistance to decay, is due either to chemical union with the cellulose, or to adsorption, which may precede chemical change in any event. Wislicenus [1920] and Koenig and Rump [1924] have argued for the latter, and there is much to support this idea [but see Schwalbe, 1920]. The complexes thus set up are probably similar to those with substances secreted by fungi which produce sap stains [Hubert, 1921]. The deposition of salts in crystalline or colloidal form takes place apparently in the same way as in stiff gels. Silica is deposited in the form of rods placed longitudinally, thus permitting the lateral swelling of the walls [Brown, 1920]. The gelatinous walls of algae play a rôle as the medium in which is deposited iron, oxyhydrate. In addition to *Leptothrix ochracea*, flagellates, blue-green and grass-green algae take part [Steinecke, 1923].

Cellulose walls are all secondary, though the usual descriptions do not conform to this view. The primary membrane appears to be more or less identical with the middle lamella, though this is undoubtedly more extensive than the primary membrane in the strict cytological sense. As usually defined, the middle lamella has properties due to its combination with calcium as pectate, a stiff, adhesive colloid. If its chemical integrity is disturbed, abnormal interchanges of ions take place between the plant and the soil, as, e.g., if Ca is replaced by K [True, 1922]. It is, therefore, important to recognize the probable rôle of the cell wall as contributory to a general result, the whole of which has hitherto been attributed to the plasma membrane alone.

Again chemical alteration of the middle lamella loosening the Ca may give rise to mucilage. Living root-cap cells are thus set free [Knudson, 1919]. Those of abscission layers [Lloyd, Sampson, 1918, Hodgson, 1918] suffer general or localized changes of the same kind, which may also occur in pathological or normal conditions, giving rise to lesions (e.g., gummosis) or lysigenous pockets, as the case may be. Such pockets of small size are common, but in some Cataceae are very extensive.

According to Odén [1917] the pectin bodies constitute a reserve of acid in undissolved condition, which, by neutralizing alkalis serves to maintain the balance of H- and OH-ions of the plant body. ′

COLLOIDAL CONDITION IN GLANDULAR ACTION

The excretion of molecularly dissolved substances need not here concern us especially. That of materials which are not miscible with water (oils,

resins) must either suffer changes (hydrolysis, saponification) under the action of enzymes, thus rendering them diffusible, or their passage from the inside to the outside of the secretory cells must be explained as interaction between colloids. This difficulty has been avoided by Tschirch by arguing that a "resinogenous layer" occurs, in this way referring the secretory activity to the outer regions of the cell wall. In the light of the work of Moenikes [1924] and others, this theory seems untenable. Moenikes has shown that the secretion first occurs as minute droplets in the protoplasm of the epithelium cells even before a vacuole has been formed. In the lumen of the resin canal (Compositae, etc.) no mucilaginous layer, in the sense of Tschirch, could be found in *Parthenium* even by Lloyd [1911] who inclined to his view. Moenikes, on the other hand, found the luminal excretion to occur at first in the form of minute droplets of the same nature as those within the protoplast, and it may be inferred that the passage through the cell wall proceeded without any change. This has never been observed, however, and the problem remains open. It is the same met with in the translocation of fats through water permeated membranes in both animals and plants, the latter exemplified in the mobilization of oil from the endosperm to the embryo [Lloyd, 1910], in which it is always the cellulose wall which offers the greater impediment, since the passage of particles or suspensoids through plasma involves little difficulty. Since, however, the cellulose wall may, if Hansteen-Cranner is right, be regarded as a deposition in a meshwork of protoplasm, at the outer limits of the protoplast, if the particles of secretion are small enough, they would meet here with less hindrance than has been supposed.

We are confronted not only with the need of explaining the passage of colloidal materials (ultrafiltration), but their collection in the lumen or other place of storage. When surface active substances are involved, their collection at the surface of the medium affords a partial explanation, but it does not seem to throw any light on the extrusion of droplets beyond that surface. If, however, this has a low surface tension (it being often an air-water interface), induced by the presence of a lyophile colloid, such as as thin a layer of mucilage as might be found in the water film on the cell-wall facing the lumen, the occurrence of droplets can be understood. This is perhaps a restatement of Tschirch's view in its applicability to the collection in the lumen of secrete, in disregard of its origin.

Resistance to Loss of Water by Evaporation and Freezing

The most insistent peril that besets terrestrial life is loss of water, either by evaporation or freezing. Whether the injury in the latter process results directly from loss of water involving physicochemical changes in the plasma [Maximow, 1914] or as is less likely, merely from mechanical injury by the ice crystals, does not affect the fact that whatever enables the cells to hold their water increases their resistance to low temperature. With regard to loss of water by evaporation the protective part played by colloids in mucilaginous plants is too familiar an idea to demand more than passing note. But in resistance to undercooling also there is evidence that the colloids (including protective colloids) of the cell play an important part. While resistance to cold, may, in some cases (e.g., in the avocado, *Persea*), be correlated with slight differences in osmotic pressure, it is more closely related to the total extractives [Panatelli, 1918]. The concentration of electrolytes indeed

is not sufficient to account for resistance to freezing. Some other factor, therefore, must be at play. That this may be retention of water by the colloids of the cell is supported by the great increase in water holding power exhibited by "hardened" plants. The tissues of hardened wheat will not lose their water under 400 atmospheres pressure [Newton, 1922]. The leaves of hardened plants lose water by evaporation much less readily than those of unhardened [Boswell, 1923]; the sap of such plants is less readily precipitated by cold[22]

According to Lewis and Tuttle, the chloroplasts lose their identity during extreme cold, regaining it again in early spring. The course of events needs and deserves further elucidation.

PERIODIC PRECIPITATION (LIESEGANG PHENOMENON)

The occurrence of periodic precipitation in tissues was first observed by Frommann in the axons of nerve fibers which had been treated with silver nitrate which reacted with the chlorides present. The appearances were explained by Boveri in 1886 on the basis of an experiment done by A. Boehm, who obtained the appearances which have now become known as "Liesegang rings" by placing capillary glass tubes filled with egg-albumin in a solution of silver nitrate.[23] Liesegang himself in 1898 described them extensively and traced analogies with certain animal structures. Küster [1913, 1917] has furnished an extensive study of such analogies seen especially in plants. The subject is intriguing and elusive. Analogies have been seen between the Liesegang rings, or bands, and the color patterns in the leaves of such plants as *Eulalia zebrina, Gasteria* and the species of *Bilbergia Cryptanthus; Dracacna, Haworthia, Aloe, Sansevecria,* etc., in seed coats, the growth zonations of stems, fungus colonies, the spiral and other thickenings of the cell wall (tracheae, tracheids), and of spheromicrocrystalline masses of starch grains and [Küster, 1913], it may be added, of the cell wall during its whole development. The layerings in the cell wall of the cotton fiber are regarded by Balls [1919] as conditioned by diurnal periodicity of growth, as A. Meyer [1895] had previously interpreted the zonation in starch grains. With regard to the latter, Küster [1913] believes to have proved that Meyer was wrong, and to have confirmed the results of H. Fischer [1902]; and, in the absence of correlation with external factors, maintains that the zonation in question is due to internal rhythm.

Such rhythm may occur during crystallization. Köhler [1915] obtained it in suddenly cooled films of sulfur, and I have repeated his experiment successfully. I have further got it in evaporating methylene blue, and in spherocrystalline bodies of cane sugar. It may be that the zonations in hailstones [Lloyd, 1916] are of this nature. Droplets of sulfur passing from the metastable into the spherocrystalline state show internal rhythm, and this is betrayed in the concentric fracture of all cryptocrystalline sulfur forms (personal observation). It seems, therefore, advisable to make a close investigation by comparison of the zonate structure of such systems with those in starch and such like organic products.*

[22] For extensive citations see Rosa, 1921; Lottermoser, 1909; Harvey, 1922.

[23] For an historical summary of this phase of our knowledge see Macallum, A. B. and Menten, M L., "On the distribution of chlorides in nerve cells and fibers," *Proc. Roy. Soc.,* **B77,** 165-193, pl. 2-4, 24 July, 1905.

* Some interesting data on the crystallization of sulphur is to be found in Vol. I, this series. Layered structures are treated by Schade, this volume, and the Liesegang phenomenon is discussed in several papers in Vol. I, including one by Liesegang. The concentric formation of hailstones is most likely due to their repeated rise and fall through a formative zone (Simpson). *J. A.*

It may be recalled that the presence of colloids is not a *sine qua non* for producing Liesegang rings [Morse and Pierce, 1903]. I have obtained beautifully regular ones by permitting ammonium sulfate to diffuse under a cover slip into weak cobalt-sodium hexanitrite. Gels especially furnish a scaffolding which prevents other than ionic and molecular movements and may retard diffusion. It may therefore be expected that the Liesegang phenomenon will display itself if the appropriate conditions are supplied in the plant. This is indicated by the studies of H. P. Möller [1922] who found that Liesegang bands are formed when silver nitrate is allowed to diffuse into the grains of various cereals, since it reacts with the chlorides already present. The different grains (wheat, barley, rye, oat and rice) behave variously, indicating specific differences in the concentrations of salts present or in the kinds of compounds present. At all events, Möller obtained rhythmic precipitation in the nucellar tissues and aleurone layer cell walls either normal to the surface of the grain or parallel thereto, according to the direction of entrance of the reagent, which in association with alcohol can enter the otherwise impermeable corky integumental layer. Otherwise, the reagent can enter only through accidental openings. Möller further obtained similar results by allowing the diffusion of the reagent to take place through an opening in the thick epidermis of a leathery leaf, and by permitting the end of a dried strip of epidermis (*Sedum maximum*) to dip into the reagent. This author concludes from his observations that the phenomenon is the true Liesegang banding and that the cell walls are colloidal.

Very striking results have also been obtained by myself [1923] incidental to an attempt to make use of sodium-cobalt hexanitrite [A. B. Macallum, 1922], for the detection of potassium salts in plants. Since the triple salt formed with potassium is white and soluble except at low temperatures, and is difficult to detect microscopically, it requires to be fixed in insoluble and visible form. This may be accomplished by ammonium sulfide. A critical use of the cobalt reagent requires, however, that every trace of the unprecipitated sodium-cobalt reagent be removed before the sulfide is applied. This, as I have found, is extremely difficult to achieve, more especially in situations where the cuticle forms inclosed spaces, as in trichomes, and when the double salt is left, the ammonium sulfide forms with it, upon diffusing into such structures, most beautiful Liesegang rings. I have been unable to obtain evidence of this behavior when cobalt-sodium hexanitrite diffuses into gelatin or agar containing potassium alone, and we should consequently not expect that it would in a tissue. Nevertheless, I have obtained a coarsely granulated precipitate in zones in the cytoplasm of trichomes, whereas the triple ammonium salt is apparently confined to the cell walls. This latter occurs also in Liesegang bands in the cell walls in the multicellular trichomes in transverse section of the *Begoniaceae*, and in the stele of roots where its limitations are supplied by the impermeable endodermis [Lloyd, 1923]. It is therefore permissible to advance the theory that substances diffusing into growing points and masses, such as young leaves, can react with other substances already present in such fashion as to produce the banding above alluded to, which really does bear a resemblance to the varieties of banding seen. The subsequent extension of the organ only spreads or distorts the pattern [Küster].

Aleurone grains.[24] These bodies are most interesting colloidal complexes containing "crystalloids" of protein and often crystals of calcium oxalate,

[24] For particulars of detail see Tunmann, 1913.

accompanied by droplets (globoids), usually one large and often many minute in size, constituting an internal phase imbedded in a ground substance [Rostovtzev, 1916]. A membrane incloses the whole. On slow access of water to a glycerol preparation, the external phase swells, bursts the membrane and sets free the globoids and crystalloids in the water. If the oil (*Ricinus* endosperm) runs gently in the glycerol, it may envelop and protect the aleurone grain from the water, so that single ones suspended in a small droplet of fat may be seen. Immediate access of water results in the formation of an emulsion of globoid in fat. If a droplet of blood be added to such preparation, agglutination of the erythrocytes occurs, followed slowly by haemolysis. If, however, a small section of endosperm is extracted by ether, freeing it of fat, and water added, the globoids become suspended in the water. If a droplet of blood is now added, hemolysis takes place immediately. That there is present a substance which emulsifies the fat, becomes apparent upon sustained observation of the fat in a glycerol-water preparation. These observations are mentioned in order to indicate that even in such non-living organic parts of the cell, substances exist side by side which require only the addition of water to set in motion a train of physical changes involving especially surface tension effects. The powerful nature of some of these substances is known, e.g., saponin, ricin, abrin. How these substances may act *intra vitam* is not known. Lipase and rennet (in the seed of *Ricinus*) are said to exist in the zymogen conditions till the onset of germination.[25] Nevertheless, an immediate action may be traced. Ether-extracted seeds which have imbibed water exhibit a far different aspect from dry ones, resulting from the disappearance of the membrane about the aleurone grain and the setting free of crystalloids and globoids. Whether the change is reversible or not we do not know.

It is not improbable that the investing membrane is nothing more than a concentration membrane. The separation out of artefacts topographically identical with natural aleurone grains on dialyzing the NaCl-extracted protein of the embryos of *Bertholletia excelsa* [Thompson, 1912] suggests this, as also the mode of swelling in oil when water is available, as I have observed.

MITOSIS

The changes which occur during indirect cell division, descriptions of which occupy an immense literature, have been, on the whole, regarded chiefly from the morphological point of view. It has, of course, been recognized by many observers that these changes are susceptible of explanation in the same manner as those which might occur in colloidal media of comparatively high viscosity, in which syneresis, vacuolization, flocculations, osmosis, imbibition, etc., and their reversals, can produce changing structural configurations. A goodly number of attempts have been made to do this, not without a measure of success. The mitotic process is, however, so complex, that such attempts have more of suggestive than of convincing value. Thus, while we may see in the segregation of chromatin to form chromosomes a reversible flocculation process, the fact demonstrated by Morgan and his students, of the existence of character-loci in these bodies, forbids a too easy acceptance of so simple an explanation—one which simply does not go far enough, however true it may be fundamentally.

[25] Green, R., "Soluble Fermenta and Fermentation," p. 390.

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Colloidal State and Physiological Function

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All living substances resemble each other in their chemical, physico-chemical, physical and microscopical properties; therefore they are all included in the same term "protoplasm."

Protoplasm contains proteins and lipoids besides many other organic compounds. It has a peculiar semifluid consistency, all transitions between the behavior of the real liquids and the solids being found in it. It is this semifluid state that gives to the protoplasm a relatively solid shape and at the same time a relatively small power of resistance to deformation; the semisolid state also corresponds to the more or less distinct existence of a visible internal structure. These properties are based chiefly on the facts, that the proteins and lipoids are colloids and that they belong to a group of colloids capable of showing a great many different appearances in the colloidal state.

It is known, that generally *sols* and *gels* are contrasted as types of the dissolved and nondissolved conditions of colloids, and *hydrophile* and *suspension* or *hydrophobe colloids* as types, which are distinguished from each other by the more or less sharply defined limits between their particles and the surrounding water. Protein may form, for instance, a hydrophile colloid sol (I), that resembles a real solution even in its ultramicroscopic behavior, but by concentration, with increasing viscosity, it is gradually changed into a hydrophile colloidal gel (II), which gains the relatively great solidity of a jelly without losing its optical homogeneity in any essential degree. Likewise the hydrophile colloidal sol of a protein may be easily changed by various means into a suspensoid colloidal sol (III), the colloidal particles of which form ultramicrons distinctly delimited against the water, and these too are able to form, on being concentrated, a jelly-like mass, a suspensoid colloidal gel (IV), which one can distinguish without difficulty from a hydrophile colloidal jelly by its distinct internal structure.

It is of great importance for physiology, that the different changes of condition of the colloids just named can be effectuated in one direction or the other by the addition of electrolytes and in this connection the effects of the salts are especially interesting, because they open the possibility of *explaining the various physiological actions of the ions as influences on the colloids of protoplasm*. One may assume that the results of the action of salts will be changes in consistency and distribution of the colloids, which may be followed by alterations of the physiological functions.[1] Especially noteworthy are the alterations that the surface colloids of the cells undergo; for on their condition is dependent the relatively undisturbed process of the reactions inside the cells and also the exchange between the inside of the cells and the outside

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[1] For fuller discussion of the subject see: Höber, "Physikalische Chemie der Zelle u. der Gewebe," I (1922), and II (1924).

world, between the protoplasm and its environment. We must further examine *electrical stimulation* from this viewpoint, because stimulation is accompanied by a redistribution of the naturally present ions in the protoplasm.

The following article will first indicate briefly, that the artificially induced changes of ion concentration in the exterior and interior milieu of the cells are connected with physiologically significant changes of the condition of the colloids. In addition it will be shown, that perhaps in the normal processes of life there exist too electrolytic reactions in the protoplasm itself, which bring about changes in colloidal state inside the cells; this suggestion is essentially based upon the *bioelectrical phenomena*.

I. Artificial Changes of the Condition of Cell Colloids Produced by Electrolytes and Their Effect on Function

1. Changes in the Colloidal State Produced by Neutral Alkali Salts

It is known that neutral alkali salts produce changes in the interaction between the hydrophile colloids and the solvent, which in the case of the sols, even in the presence of relatively small salt concentrations, become apparent as alterations in osmotic pressure and viscosity; in the case of the gels and in the presence of large concentrations of salt as alterations of imbibition ("Quellung") and dehydration ("Entquellung"). The anions differ from each other as to the intensity of their action in a characteristic series, which is known as the *Hofmeister series*,[2] i.e., in the series: SO_4, Cl, Br, NO_3, I, SCN. The corresponding series of cations is more variable, especially with change of reaction; if the reaction is neutral, the gradation of influence is usually given by the series: Li, Na, Cs, Rb, K, whereas in acid or alkaline reaction the cations are arranged according to their atomic weights: Li, Na, K, Rb, Cs; the proteins, for instance, are most easily coagulated in acid solution by Li, least by Cs, at alkaline reaction the series is reversed, Cs being strongest and Li the least potent,[3] the series of cations thus running in one or the other direction under different circumstances; therefore the series valid for neutral reaction can be conceived as an intermediate arrangement, as a "transition series" ("Uebergangsreihe"). Likewise it is true for anions, that one end of the series or the other gives the maximum effect. For instance with egg albumin it is only necessary to increase or diminish the salt concentration to produce a reversal of the series as indicated by the temperature of coagulation.[3] Making an alkaline solution acid likewise causes a reversal of the series.

Now the action of the alkali salts on all kind of physiologic objects differs in the same characteristic manner; we meet the Hofmeister series of anions, the transition series of cations and the reversal of the arrangement of the ions. The alkali salts are not only supposed merely by analogy to exercise their effects on the cell colloids, but such influences can be demonstrated directly in various cases.

Let us begin with experiments, in which the physiological action of the alkali salts is accompanied by the *escape of a colored substance* from inside the cells. In order to demonstrate this, a good material is found in the pig-

[2] F. Hofmeister, *Arch. exper. Path. Pharm.*, 25, 13 (1888); 28, 210 (1891).
[3] Hober, *Beitr. chem. Physiol. Path.*, 11, 35 (1907).

mented eggs of certain sea urchins or in the pigmented larvae of annelids and especially in the red blood corpuscles, whose hemoglobin can be separated from the stroma by the process of *hemolysis*. If we bring the blood corpuscles into weakly hypotonic solutions of the different alkali salts, we find, that the hemoglobin leaves the cells with increasing velocity in accordance with the series: $SO_4 < Cl < Br < NO_3 < SCN < I$ and Li, Na $< Cs < Rb < K$;[4] but if we put the corpuscles into sodium hydroxide or into saponin in presence of the salts, we cause the anions to hemolyse in the reverse series: $SO_4 > Cl > Br > I > SCN$.[5] In the same way the pigment is lost by the eggs of Arbacia, if they are allowed to stay in isotonic solution of KCl or $NaCl$ instead of sea water; they lose their pigment more quickly in $NaCl$ than in KCl, and by varying the anions the increasing rapidity of the escape of color is given by the series: $Cl < Br < NO_3 < SCN$, I.[6] Thus the cells behave nearly like colored droplets of gelatin, that are dissolved in increasing degree on swelling in the different solutions. Especially the action of hydrogen and hydroxyl ions gives strong evidence for the hemolysis being caused by changes of colloidal state as produced by the ions. For instance, the influence exercised on the hemolysis by temperature or by alcohol in presence of different quantities of acid or alkali has been investigated,[7] and it has been stated, that the maximum hemolysis occurs at $pH = 3$ and $pH = 10$; at these same reactions the dissolution of the blood corpuscles is followed by a visible flocculation in the fluid as a consequence of the coagulation of colloids. From these experiments and other observations we may conclude, that the lysis is introduced by the disaggregation of the colloids in the body of the cells.

We may now suppose the colloids of the cell surface will be greatly altered by the action of ions. But there is still another reason for the same hypothesis, i.e., the observation, that the salts change the *permeability of the cells* to substances that are dissolved in their environment, in the same characteristic manner as they act on the colloids. This has been demonstrated by measurement of turgor and of plasmolysis of *plant cells*.[8] For instance, plasmolysis was measured by the velocity of deplasmolysis that occurs in the different hypertonic salt solutions. Plant cells under ordinary circumstances in their natural surroundings do not permit salts to pass the surface at all or at least only very slowly,[9] but in the solutions of the pure alkali salts they soon become permeable. The velocity of this change increases in accordance with the series of anions: $SO_4 < Cl < Br$, $NO_3 < I$ and with that of the cations Li $< Na < K$. A similar influence is exhibited by the salts on the *chemical changes in plant protoplasm;* an instance is the intensity of fermentation of yeast.[10] In regard to the question of the injury of the surface colloids by salts a *disturbance of growth* must further be mentioned. This phenomenon is caused by the effect of salts on the young roots, their rigid cell walls being destroyed by imbibition and mucous liquefaction, especially in the zone of stretching; this is brought about by the presence of pectins and lipoids in the framework of cellulose, which takes up abnormously large amounts of water under the influence of the salts.[11]

[4] Höber, *Biochem. Z.*, 14, 209 (1908).
[5] Port, *Deutsch Arch klin. Med*, 99, 259 (1910); Miculicich, *Centralbl. Physiol.*, 24, 523 (1910); Ternuchi, *Comm. de l'Institut serother l'État danois*, 3 (1909).
[6] Lillie, *Am I. physiol*, 26, 106 (1910); 24, 14 (1909).
[7] Jodlbauer u Haffner, *Pflugers Arch.*, 179, 121 (1920); 196, 15 (1922).
[8] Osterhout, *Science*, 35, 112 (1912); Fitting, *Jahrb wiss Bot*, 56, 1 (1915); 57, 553 (1917); Tröndle, *Arch. sciences phys nat.*, 45, 38 (1918); Kahho, *Biochem. Z.*, 123, 284 (1921).
[9] Netter, *Pflugers Arch.*, 198, 225 (1923).
[10] Boas, *Biochem. Z.*, 117, 166 (1921)
[11] Hansteen-Cranner, "Meldinger fra Norges Landbrukshoiskola," 1922.

The effects of alkali salts on *ciliated epithelium and cilia* may be compared more or less with the processes of hemolysis, or more generally cytolysis. The motion of cilia of the gill epithelium of Mythilus or the ciliated larvae of Arenicola or of the mucous membrane of the throat of frogs, is in each case injured according to the anion series: $SO_4 < Cl < NO_3 < Br < I$ and the cation series: $K < Rb < Cs < Na < Li$. That these processes are caused by a change of condition of the colloids, is proved by the observation that the cilia of the Arenicola are directly liquefied by pure salts, especially the halides of Li and Na.[12] The movements of the spermatozoa are also influenced, the anions working according to the usual Hofmeister series, the cations are effective in similar series in Rana temporaria and guinea pig; but it is peculiar that, while in Rana temporaria the series is: $Rb > K > Na > Cs$, Li, in guinea pig it is: K, $Rb < Na < Cs$, Li.[13]

As a last example of changes of colloidal state by alkali salts we may discuss their influence on *muscles*. If we put the latter in isotonic solution of the alkali salts, the *irritability* is preserved to a greater or lesser degree. Among the cations K and Rb paralyse the muscles rapidly, Cs slower and Li still slower, while in Na the irritability is best preserved. We therefore find the following series: $K > Rb > Cs > Li > Na$.[14] The corresponding anion series is under certain conditions $SO_4 > Cl > Br > NO_3 > I > SCN$.[15] The changes in irritability parallel other changes in the *electrical behavior of the muscles*.[16] Salt-demarcation currents ("Salzruheströme") are produced, if one moistens one end of the muscle with Ringer solution and then puts in it a nonpolarizable electrode, while the other end is moistened with an isotonic salt solution, in which rests a second electrode. The EMF of these currents is of varying strength in accordance with the series of anions and cations already named, and the EMF developed is in proportion to the change of irritability of the muscle. These salt effects are reversible; for instance the negativity, which is generated by KCl, disappears with the removal of the salt. Now it is to be remembered, that normal stimulation produces a temporary negativity too, while injury causes a lasting negativity such as would be due to perforation of the surface membrane. Therefore we may assume, that the salts produce the negativity by causing an imbibition of the surface colloids and in this way increasing the permeability to ions. This may be compared to the effect of injury and differs only by the fact, that it is not reversible. We may further assume, that normal stimulation produces an increased permeability owing to a reaction of the electrolytes within the cells and the surface colloids.[17] That stimulation and increased permeability are closely related, has been shown in many ways.[18]

2. Variations in Colloidal State Produced by Changes in Electric Charge

It is well known that colloidal particles usually carry either a positive or negative electric charge, and that the stability of their permanent dispersion is

[12] Lillie, *Am. J. physiol.*, 17, 89 (1906); 24, 459 (1909); Hober, *Biochem. Z.*, 17, 518 (1909).
[13] Gellhorn, *Pflügers Arch.*, 193, 555 and 576 (1922).
[14] Overton, *Pflügers Arch.*, 105, 176 (1904).
[15] C. Schwarz, *Pflügers Arch.*, 117, 161 (1907).
[16] Höber, *Pflügers Arch.*, 106, 599 (1905).
[17] See also Mond, *Pflügers Arch.*, 203, 247 (1924).
[18] Gildemeister, *Pflügers Arch.*, 162, 489 (1915); 200, 278 (1923); Ebbecke, *Pflügers Arch.*, 190, 230 (1922); Osterhout, *Science*, 45, 97 (1917); Embden & Adler, *Z. physiol. Chem.*, 118, 1 (1922); Ebbecke & Hecht., *Pflügers Arch.* 199, 88 (1923); Banus, *Pflügers Arch.*, 202, 184 (1924).

partly due to this EMF. The electrical charges may be neutralized by the addition of suitable ions. Then the sols lose their stability, the discharged particles coalesce, flocculate and precipitate. Especially suitable for the neutralization of positive particles are OH ions or bivalent or trivalent anions, while for the discharging of negative particles H ion or bivalent or trivalent cations are best. A neutralization of the electrical charge may produce even a flocculation within the jellies of hydrophile colloids.

The cells of the body, just like colloidal particles, carry electric charges. The causes of these charges are manifold. In part they arise from the Donnan equilibrium,[19] in part from a selective permeability for anions;[20] adsorption of ions at the surfaces also plays a rôle,[21] and finally the charges depend on the nature of the colloids which are adsorbed at the surfaces of the cells from the surrounding fluids; for example red blood corpuscles adsorb colloids from the plasma. As can easily be proven by cataphoresis, the electric charges of cells are mostly negative.[22] Therefore the neutralization or even reversal of the charge is successfully accomplished by the H ions or polyvalent cations, like those of the rare earths (Al, La, Ce). As the isoelectric point is approached, the cells begin to clump like the particles of a sol, finally they agglutinate, and therefore a quicker *sedimentation of the cells* takes place.[23]

The colloids take part in this agglutination at least to the extent that they are adsorbed at the surface of the cells, whose charge is dependent of the ions which may be present in the surrounding fluid. This has been clearly shown in the case of red blood corpuscles. The speed of agglutination and sedimentation is in proportion to the ratio of globulin and albumin in serum or plasma; the most marked agglutination takes place with fibrinogen.[24] This is at least partially dependent of the different isoelectric points of the various protein bodies in the serum. The negative charge of the red blood corpuscles is diminished by the adsorbed proteins and the more so, the closer the isoelectric point of the particular proteins approach the neutral point, which nearly coincides with that of the reaction of the blood.[25] The isoelectric point of albumin is more acid than that of globulin; therefore the latter more easily agglutinates suspensions of blood corpuscles. This relationship was proven by measurements of *cataphoresis* of blood corpuscles in solution of the salts of the rare earths in various concentration. The smaller the negative charge of the blood corpuscles, that is the nearer the reaction is to the isoelectric point, the smaller is the concentration of La required to completely neutralize the charge.[26] Red blood corpuscles coated with fibrinogen need a much smaller concentration of La ions for neutralization than these coated with albumin. An example is given in the following table. The plus and minus signs indicate the kind of electric charge, while the number of plus signs gives the magnitude of the charge.

[19] E. J. Warburg, *Biochem. J.*, 16, 153 (1922).

[20] Koeppe, *Pflugers Arch.*, 67, 189 (1897); Hamburger & Van Lier, *Arch. Physiol.*, 1902, 492; Rohonyi, *Kolloidchem. Beihefte*, 8, 337 (1916); Ege, *Biochem. Z.*, 107, 246 (1920) and others.

[21] Haffner, *Pflüger's Arch.*, 196, 15 (1922). [An important probability in some cases may be *electro-version*, whereby there tends to be presented to the milieu those portions or fields of a particle, or even of a molecule, which have a charge opposite to that of the milieu interface. *J. A.*]

[22] Lillie, *Am. J. physiol.*, 8, 273 (1903); Hober, *Pflugers Arch.*, 101, 607 (1904); 102, 196 (1904).

[23] Bechhold, *Z. physik. Chem.*, 48, 385 (1904); Neisser & Friedemann, *Münchn. med. Wochenschr.*, 1904, 465; Teague & Buxton, *Z. physik. Chem.*, 57, 76 (1906); Kozawa, *Biochem. Z.*, 60, 146 (1914); Mines, *Kolloidchem. Beihefte* 3, 191 (1912).

[24] Fahraeus, *Hygeia*, 1918; *Acta medica scand.*, 55, 1 (1921); v. Oettingen, *Biochem. Z.*, 118, 67 (1921); Starlinger, *Biochem. Z.*, 114, 122 (1921) and 122, 105 (1921).

[25] Hober & Mond, *Klin. Wochenschr.*, 1922, Nr. 49, Kanai, *Pflugers Arch.*, 197, 583 (1922); Mond, *Pflügers Arch.*, 197, 574 (1922); Ley, *Pflügers Arch.*, 197, 599 (1922).

[26] Fahraeus, *Biochem. Z.*, 89, 355 (1918); Linzenmeier, *Pflugers Arch.*, 181, 169 (1920) 186, 272 (1921).

Fluid Containing	Speed of Sedimentation	Concentration of La				
		m/1250	m/1000	m/750	m/500	m/400
Pseudoglobulin	51	±	+	++	++	++
Equal parts of Pseudoglobulin and Albumin	41	—	±	+	+	++
Albumin	1	—	—	±	+	+

The sedimentation is fastest in pure solution of pseudoglobulin, because the electronegative charge is smallest. In addition it should be mentioned that globulins and especially fibrinogen flocculate more easily than albumins; for this reason too suspensions of red blood corpuscles coated with globulin are less stabile than those coated with albumin.

The neutralizing and flocculating influence of blood proteins probably plays an important rôle in *phagocytosis*.[27] Leucocytes and carbon particles, when suspended in solution of various proteins, stick together best in fibrinogen solution and least in solution of albumin. This agglutination between leucocytes and carbon, the superficial contact of carbon particles and leucocytes, probably is the first stage of phagocytosis. We can therefore understand how the increased globulin fraction occurring in infection diseases and inflammatory processes is a purposeful reaction of the organism, since the course of the disease is greatly influenced by a well developed phagocytosis.*

Disregarding the protein bodies, which may be adsorbed at the surface of the cell from its environment, it seems just to assume that the neutralizing influence exerted on the cells by very active cations like H or La is brought about by their reaction with the colloids, which as proteins and lipoids are present in the surface membranes of the cells. The agglutination of red blood corpuscles occurring in the region of the isoelectric point is usually followed by hemolysis. This seems to indicate that an "internal agglutination" takes place—a flocculation of the colloidal particles of the protoplasm—so that the cells are disorganized and destroyed.[28] Such an internal agglutination may also explain the peculiar behavior of muscle on being poisoned with the salts of the rare earths.[29] If the salts are in great or small concentrations, the muscles recover on their removal, but medium concentrations produce an irreversible injury. This phenomenon may be explained by the fact that at the medium concentrations an isoelectric flocculation takes place, destroying the internal structure of the cell, while at the high concentration the colloids are held in solution by reversal of the charge and peptization.

3. CHANGES OF COLLOIDAL STATE FOLLOWING THE PASSAGE OF ELECTRIC CURRENT

Instead of changing the colloidal state by non-physiologic salts or physiologic salts in abnormal concentration, the same effect may be produced by a change in the ionic environment on the passage of an electric current. If an electric current is passed through a salt solution, in which lies a colloidal diaphragm, there occurs a difference of ionic concentration in both surfaces

⁷ Hober & Kanai, *Klin. Wochenschr.*, 1923, S. 209; Kanai, *Pflügers Arch.*, **198**, 401 (1923).
* See paper by H. A. Abramson in this volume. *J. A.*
²⁸ Michaelis & Takahashi, *Biochem. Z.*, **29**, 439 (1910); Mines, *Kolloidchem. Beihefte* **3**, 191 (1912); Kozawa, *Biochem. Z.*, **60**, 146 (1914).
²⁹ Hober & Spaeth, *Pflügers Arch.*, **159**, 433 (1914).

of the diaphragm. This phenomenon is due to a difference of the speeds of migration of ions in the substance of the diaphragm from that in the aqueous solution.[30] The ions of water are especially important because of their great reactivity with regard to colloids. The OH ions accumulate at the anode and the H ions at the cathode.

These same changes in ionic concentration occur at the surfaces of living protoplasm. If a current is passed through the cells of the stalk of *Tradescantia*, whose cell-sap contains a reddish-violet dye, the sap becomes green at the anode and red at the cathode, due to changes of reactions at both electrodes.[31] The changes in ionic concentration apparently entail variations in the colloidal state, which distinctly manifest themselves in differences in permeability. If a weak alternating current or a direct one, whose direction is changed every 5-10 seconds, is passed through the freshly cut stem of a plant, there occurs a marked and reversible decrease of resistance, as well as a reversible diminution of turgor. The latter makes itself manifest most strikingly by a wilting.[32] Chains of *Spirogyra* may float for weeks in a solution of acid dyes (Fuchsin S, Cyanol), to which they are impermeable, without taking any of the dye, but if an electric current is passed through the *spirogyrae*, they readily absorb the dye. The cells, however, remain entirely uninjured.[33] In connection with what was said above, these changes in permeability may be most easily understood as changes in the colloids, produced by variation in the environmental ions.

4. COLLOIDAL CONDITION IN SALT MIXTURES

We have already cited numerous examples in which salts disturb the functioning of cells and tissues. It is well known that salts form a part of the physiological environment of cells. Salt solutions produce disturbances when they consist of non-physiologic salts or physiologic salts in abnormal concentration. The importance of a normal mixture became known chiefly by the great advantage of Ringer's solution.[34] While many functions are disturbed in a pure NaCl solution, just a small addition of KCl and CaCl₂ is enough to return normal conditions. Sea water is above all others a normal salt solution or, as *Loeb* called it, *a physiologically balanced salt solution*. Any change in the constituent salts injures the organisms that live in the sea.[35]

A study of the deeper significance of these mixtures teaches us, that their effect depends for the greater part on the fact that they maintain the normal colloidal state of cells and tissues. Therefore we will first show the *effect of salt mixtures on colloids*. The most important fact in this regard is the *antagonistic action, which monovalent and polyvalent cations exert* on the negatively charged colloids. For example if bivalent or polyvalent cations are added to a solution of a salt of monovalent cations, their effect is not to increase the salt effects on solubility or imbibition, but, on the contrary, to decrease them. This phenomenon can be shown successfully only in such colloids that are more or less hydrophile in character.[36] A given sulfur sol

[30] Bethe & Toropoff, *Z. physik Chem* **88**, 686 (1914) and **89**, 597 (1915).
[31] Bethe, *Pflugers Arch* **163**, 147 (1916).
[32] Ebbecke & Hecht, *Pflugers Arch*, **199** 88 (1923)
[33] Hober & Banus, *Pflugers Arch*, **201** 14 (1923); *Banus, Pflugers Arch*, **202**, 184 (1924).
[34] Ringer, S., *J physiol*, **3**, 380 (1882); **4** 29, 222 & 370 (1883); **7**, 118 & 291 (1886).
[35] C. Herbst, *Arch. Entwicklungsmech.* **5**, 650 (1897); **7**, 486 (1898); **11** 617 (1901); **17**, 306 (1904); Loeb, *Pflugers Arch.*, **97**, 394 (1903), **101**, 340 (1904), Wolfgang Ostwald, *Pflugers Arch.*, **106**, 568 (1905).
[36] Freundlich & P. Scholz, *Kolloidchem. Beihefte*, **16**, 234 & 267 (1922).

may be flocculated by 1100 millimols of 0.5 Li_2SO_4 or by 0.06 millimol of $CeCl_3$. If $CeCl_3$ is added to 65 per cent of the Li_2SO_4 (that is to 736 millimol), then instead of the addition of 35 per cent of the $CeCl_3$ (0.02 millimol), 9 millimol are necessary to produce flocculation, that is 15,000 per cent of the original value. The same phenomenon obtains in other cases.

We may approach closer to the actual physiological relations in the following study.[37] The cell membrane of the sea alga *Chaetomorpha* is formed of numerous lamellae of cellulose and an interlamellar substance. If the membranes are cut so that the protoplasm flows out and if one then brings the membranes into an isotonic NaCl solution, they absorb water rapidly. This swelling may be hindered by the addition of bivalent cations to the NaCl solution, as is shown in the following experiment:

m/2 NaCl, to which are added the following amounts of $ZnSO_4$......	0	m/1000	m/200	m/100	m/40	m/20	m/10	m/5	m/2
Membrane thickness at the end of 3 hours.........	10	7	4	3.5	3.5	3.5	5.5	10	15

Thus with a given rather small ratio of $ZnSO_4$ to NaCl, the membrane thickness is at a minimum. This effect of ions is analogous to the above-mentioned behavior of Li_2SO_4 and $CeCl_3$ on sulfur sol, but instead of increasing solubility we have here a dehydration.

Similar relations obtain for living cells. Best known is the example of the fertilized eggs of Fundulus.[38] No development takes place in pure isotonic NaCl solution, until we add definite small amounts of bivalent cations of Mg, Ca, Sr, Ba, Ni, Mn, Zn, Pb, UO_2 or of trivalent Al and Cr. Examples are found in the following table:

Component Salts		Per Cent Development
5/8m NaCl, without admixture..................................		0
"　　　100 ccm. +	m $BaCl_2$　　2 ccm..........	90
"	m $MgCl_2$　　2 "	75
"	1/8m $CoCl_2$　　2 "	88
"	1/128m $ZnSO_4$　　8 "	75
"	1/16m $MnCl_2$　　8 "	55
"	1/8m $NiCl_2$　　4 "	5
"	1/64m $Pb(CH_3COO)_2$ 17 "	17

Cilia of marine invertebrates behave in a similar manner,[39] and here the assumed influence of salt mixture on colloids makes itself manifest by the fact that in them the cilia retain their normal form, while they are liquefied in a pure NaCl solution. In a similar manner the fine gelatinous *membrane of the egg* of Asterias Forbesi dissolves, when the egg is taken out of the sea water and put into isotonic NaCl solution, while it is unchanged in mixtures of NaCl with Ca, Mg or Al.[40]

Experiments on the effect of complex cobalt salts on *frog muscle* [41] are

[37] Kotte, *Wissenschaftl. Meeresuntersuchungen* N.F., **17**, 118 (1914).
[38] Loeb, *Am. J. physiol.*, **6**, 411 (1902); *Pflügers Arch.*, **88**, 68 (1901).
[39] Lillie, *Am. J. physiol.*, **10**, 419 (1904); **17**, 89 (1906).
[40] Lillie, *J. gen. physiol.*, **3**, 783 (1921).
[41] Höber, *Pflügers Arch.*, **166**, 531 (1917).

especially convincing in affording proof of the influence of valency in balancing solutions. The muscles are paralysed when the KCl content of the ordinary Ringer solution is raised from 0.02 per cent to 0.06-0.07 per cent. Probably this phenomenon is the result of a too great liquefaction of the surface membrane of the muscle fibres. This paralysing influence may be compensated by adding various cobaltoammonia salts just as well as by increasing Ca, supposing that the cation of the complex salts is bivalent or polyvalent. The paralysing effect of K can be counteracted by $[Co(NH_3)_6]^{...}$, $[Co(NH_3)_5NO_2]^{..}$ and $[Co(NH_2.CH_2.CH_2.NH_2)_3]^{...}$, while $[Co(NH_3)_4(NO_2)_2]^{.}$ and $[Co(NH_2.CH_2.CH_2.NH_2)_2(NO_2)_2]^{.}$ have no such effect.

The valency rule of the ions is followed too in influencing the diffusion of hemoglobin from red blood corpuscles, the movement of spermatozoa, the permeability of plant cells, etc.[42] But it is not in every case that the physiologically important Ca can be replaced by other bivalent or polyvalent cations. There are on the contrary many physiologic functions, for which Ca is either essential or can be replaced only by the other alkali earths, Cr and Ba. Seeds of plants not only grow in a mixture of Na, K and Ca, but also when Ca is replaced by Sr or Ba; but Ca can not be replaced by the salts of the heavy metals.[43] In contrast to the fertilized eggs of Fundulus, for the adult fish Ca may be replaced only by Sr or Mg.[44] Both the indirect irritability of a muscle-nerve-preparation and the influence of the vagus on the heart disappear on the removal of Ca, but return on the addition of Sr or Ba.[45] The same obtains for the stomach.[46] In all these cases a direct chemical interpretation seems better than one of a salt effect on colloids. But many colloids, especially those that are hydrophile, behave in the same way. It is well known that even small concentrations of the heavy metal salts cause irreversible precipitations in protein solutions. On the other hand not only is the concentration of the alkali earths necessary to produce precipitation as great as that of the alkali salts, but the reaction is also more or less reversible. Certain kinds of gelatin are jellified more easily when to NaCl is added Ca, Sr, Ba, Mg or Co, Mn, Ni, but this action is inhibited by Cu, UO_2, Ce and Cd.[41] This grouping of ions agrees with the fact that Co, Mn and Ni are more similar to the alkali earths and have not as pronounced heavy metal characteristics as the other members of the group of bivalent cations.

On the whole we may assert that the physiologic significance of the maintenance of ionic equilibrium lies on the fact that only a definite mixture of monovalent and polyvalent ions can guarantee the maintenance of a definite physiologic colloidal state.

II. NORMAL FUNCTIONAL CHANGES IN CELL COLLOIDS

In Part One, many examples have been given where changes in the cell colloids, made manifest by changes of function, have been produced artificially by suitable electrolytes. It seems that changes in colloidal state may be called forth under normal condition by variations in ionic concentration inside the

[42] Hober, *loc. cit.;* Gellhorn, *Pflügers Arch.,* 193, 576 (1922); Netter, *Pflügers Arch.,* 198, 225 (1923); Wiechmann, *Pflügers Arch.,* 182, 74 (1920).
[43] Osterhout, *Univ. Cal. Publ. Botany,* 2, 317 (1907).
[44] Loeb, *Am. J. physiol.,* 3, 327 & 383 (1900).
[45] Locke, *Centralbl. Physiol.,* 8, 166 (1894); Overton, *Pflügers Arch.,* 105, 176 (1904); Mines, *J. physiol.,* 42, 251 (1911) & 43, 467 (1912); Hober, *Pflügers Arch.,* 182, 104 (1920); Schiff, *Recueil mem. physiol.,* I, 653, Lausanne, 1894; Busquet & Pachen, *J. physiol. path. gen.,* 11, 807 & 851 (1909).
[46] Hober, *Pflügers Arch.,* 182, 104 (1920).

cell. We have seen that on direct electric stimulation of plants there occurs a great though reversible increase in permeability, which may well be caused by changes in ionic concentration. The latter is seen by the decrease of resistance to electric currents and the increase of diffusion, for instance, of colored substances. The same may be shown for the skin of the frog.[47] But the skin or plant cells react in this manner not only on electrical stimulation, but also on mechanical stimulation. The skin reacts too on the indirect stimulation through the nerves. The resistance of the skin to a direct current, as well as the facility with which it becomes polarized, may be greatly decreased also by reflex stimulation.[48] Further we saw that the application of various salts produces demarcation currents in muscle, the magnitude and direction of EMF developed being dependent on the particular salt.[16] The cations and anions produce these effects in the same order in which they change the dispersion of hydrophilic colloids. Therefore the assumption of a change in permeability of the surface membrane has been taken as the foundation, on which the theory of the salt-demarcation current has been built. Changes in electric condition parallel those in irritability. The normal process of excitation, i.e., the action current, is similar to the salt-demarcation current in so far as it is reversible, the normal current-free condition being reëstablished on the removal of the unphysiologic salt. All these observations lead to the assumption that on stimulation, an electrolytic reaction takes place within the cell, which is closely bound up with changes in the colloid state, and which temporarily increases the permeability of the stimulated part. The occurrence of such electrolytic reaction has not yet been demonstrated.

[47] Gildemeister, *Pflugers Arch*, **162**, 489 (1915); **200**, 278 (1923)
[48] Ebbecke, *Pflugers Arch*, **190**, 230 (1921); **195**, 320 (1922) [In considering the behavior of so large a mass of matter as a bit of skin-surface, we must take into account its super-molecular and super colloidal structure and mechanism Thus with skin we have an interesting and complex system—see A. Krogh, "The Anatomy and Physiology of Capillaries," Yale Univ. Press, 1922. *J. A*]

Colloid Chemistry and Internal Medicine *

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Every basic advance in the field of natural science is of significance to the physician. This is especially true of the recently developed science of colloid chemistry. Colloids are present in every tissue of the human body, and the phenomena of life are invariably associated with matter in the colloidal condition. The ancient riddle concerning the fourth state of aggregation, "cell substance," has nowadays found its main answer in the realization of the fact that each cell, in a physico-chemical sense, constitutes a microheterogeneous system, separated from the exterior world, in which a large number of gel- and sol-like masses are so interrelated with water and true solutes, that the colloid behavior dominates the cell characteristics both as a unit and in detail.

A special feature of the colloidal condition, of the highest importance in life processes, is the enormous development of surface. There is profound significance in the expression: "Life is a struggle for free surface." In the vegetable kingdom the struggle for surface development is outwardly visible; the tree with the gigantic surface of its foliage represents this type. With animals the same tendency toward surface development is present, only here the organs of interchange of energy—in order to make it possible for the animal to move about--are developed inwardly, as is indicated by the microscopic structure of the lungs, the kidneys, and all glandular organs.

Still more important to life than this order of surface development is that in the zone of ultramicroscopic dimensions, such as is involved in the colloidal dispersion of substances in cells and tissues, whereby 1 gram of dry substance may present as much as thousands of square meters of surface. In the world of these "neglected dimensions" (Wolfgang Ostwald), lies a good part of the mystery of life. Life without development of energy is unthinkable. Free energy, transmissible into work, arises only at interfaces where potential may arise and remain active. The machines of industry have for the most part rigid exterior frames. With the higher animals and man the rigid parts, the bones, are mainly buried in the interior of the organism as a mechanical support; the energy is obtained from the semi-soft, colloidal tissues.

In muscles there are active the specific energies of colloids, which produce changes of form and volume. The existence of enormous internal surface facilitates in the highest degree the use of the smallest masses, the tiniest spaces; for the development even of great strength, no heavy mass is needed. Because of these advantages, the "swelling—and electrocapillary—motors of the organized world" (H. Freundlich) such as are found in muscles, possess, as colloid machines, an adaptability to the demands of life unequaled in

* Translated by Jerome Alexander.

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technology. Furthermore by adsorptive concentration or by gradual relinquishment of adsorbed material, colloids can bring about changes in concentration in the external solution, and thereby, as is the case with gland cells, accomplish the work of secretion against osmotic pressure.

Finally because of ion adsorption at the protoplasm/liquid interface, the colloids of the protoplasm have there an electric charge which underlies the development of bioelectric currents, such as are seen in the nerves and similarly also in the cells, as a conductivity phenomenon or protoplasmic activity. The three main kinds of cellular activity, the mechanical work of the muscles, the work of secretion of the glands, and the electrical work of the nerve cells, originate therefore in colloids. A detailed consideration of the nature of the processes here involved belong to the field of colloid-chemical physiology and will be considered elsewhere in this book.

EUCOLLOIDALITY

An essential preliminary to the normal functioning of the cells is a certain optimum degree of colloidality ("Eukolloiditat," H. Schade). Normal functioning of the cell involves variations in the colloidal condition, grossly expressed in the physiological condition. Disease leads to more extensive changes in the colloidal behavior of the cells and the tissues. Nothing injures the ability of cells to function so much as inroads upon their colloidal integrity. Cells can be cut, squeezed, or even rubbed in a mortar with quartz sand until they are unrecognizable, or the cell content may be extracted as a mush; but there always remains part of its function, especially of its enzymic capacity. There is, however, immediate cessation of all cell function, as soon as the protoplasm loses the individuality of its colloidal condition, even although the cell is entirely uninjured from a microscopical point of view. Even moderate heat, the moment it brings about a colloidal change, permanently destroys all function. Only while in a closely defined range of colloidality characteristic of its physiology, can the cell normally perform its functions.*

The higher and more delicate the cellular activities, the more important is it to protect the cell colloids against injurious influences. The most serious disturbance is that threatened by addition of foreign ions to the surrounding medium, by changes in osmotic pressure or in temperature. As we learn from comparative physiology, the history of evolution shows that as advance is made in the animal series, there is acquired increasing protection against these disturbances.

The maximum development of protection occurs in man. With a completeness approaching the marvelous, we find in man a stabilization of the ionic constitution of the blood and of the tissue juices, a stabilization of the osmotic pressure, and a stabilization of the temperature of the interior of the body. A large amount of work is continually performed to maintain these constant values, the constancy of the H-OH ion, and Na-K-Ca-ion concentration, the constancy of the osmotic pressure, and the constancy of the temperature. Many important organs, the kidneys, the lungs, the circulatory system, the connective tissue, the skin, the sympathetic nervous system, etc., coöperate in a delicately balanced mutual relationship in order to protect the colloidal integrity of the cells; and in cases of emergency the stomach, intestines,

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* This range seems to correspond with the *zone of maximum colloidality* described by Alexander in Vol. I of this series. *J. A.*

salivary glands and other parts, as far as possible, also lend their supporting activity.

Wide fields of research in internal medicine are, in a general way, outlined here. Even this cursory sketch indicates that there is involved an entirely new objective in medical research and thought. The physico-chemical investigation of the human body extends far beyond the cellular-pathological concept of Virchow. As is the case with all other branches of biology, medicine, thanks to the quickening influences of colloid chemistry, is on the verge of a new era (H. Schade).

As soon as we are able to disentangle its character from the great mass of data already existing in the field of internal medicine, to which the way was paved by the advances and revolutions of colloid chemistry, it is most essential that the material be classified from the broadest possible point of view. The problem of the physician is threefold: it is expressed by the three words *Diagnosis* (recognition of disease), *Etiology* (understanding the cause of disease), and *Therapy* (curing disease). The application and use of colloid principles have aided materially in all three fields.

DIAGNOSIS

The significance of colloid chemistry in diagnosis may be readily made clear. Almost all methods used by colloid chemistry in the investigation of fluid systems, have already been applied in diagnosis. We have made determinations on the widest variety of body fluids obtained in health and disease especially of the blood, urine, bile, saliva, gastric juice, intestinal secretion, the spinal fluid, in pus, in edema, etc. The delicacy of the recognition of differences in the properties of these fluids is considerably increased.

As a first instance we may take the application of Zsigmondy's gold number method to the examination of spinal fluid: in a modification adapted to the physician's requirements, this method not only gives valuable information in distinguishing between tubercular and purulent inflammation of the meninges, but also is useful in recognizing the early stages of brain syphilis. Colloid chemical flocculation reactions of the blood serum (e.g., the Sachs-Georgi reaction and the Meinecke reaction), have become of the greatest practical significance in the recognition of general syphilis, supplanting the well-known Wassermann reaction, which they surpass in many respects.*

In testing the presence of ferment activity in serum or other body fluid, the application of optical methods (Abderhalden) and of surface tension measurements (Michaelis) have been especially useful. In determining the appearance of the first traces of biliary acids, measurement of surface tension has also proven efficient in comparison with the Haykraht test (Lepehne).** The determination of viscosity (Hess, Determan, etc.) has acquired great practical significance, e.g., in recognition of the resistance passages of the blood through the capillaries.

But the medical application of colloid chemical methods is not confined to fluid systems. In the gels of the cells and the tissues, colloid chemical methods have also aided in refining and directing investigation. Application of elastometry during life (Schade) makes it possible to recognize with a

* See paper by Dr R. L. Kahn in this volume. *J. A.*
** A traditional folk method of testing for gall bladder affections was to sift flowers of sulfur on the urine. The presence of much biliary substance makes the urine wet the sulfur (lowering of surface tension), and the sulfur sinks. In normal urine it floats. *J. A.*

sensitiveness heretofore unobtainable, an edema, or in other words a disease of tissue expressing itself in a mechanical colloidal manner; and its progress may be quantitatively followed. The method of Heilbrunn (among others) which measures the displacement of the cell nucleus (which has a higher specific gravity) upon centrifugation of the tissues, should arouse general interest, because it brings to light changes in the mechanical behavior of the colloids in the interior of the individual cell.*

In addition, many other colloid methods have been used medically in the investigation of the behavior of protoplasm; e.g., testing for the appearance of Brownian motion in osmotic swelling of the salivary cells; testing transparency, that is, degree of turbidity; testing the degree of swelling, or else swelling capacity; testing hardness, flexibility, tenacity, etc.

The technic of histological staining, which has long been of the greatest use in diagnosis, is essentially applied colloid science, although its medical development was purely empirical; only recently has the application of physicochemical knowledge permitted a more profound insight in this field. Thanks to scientific colloid chemistry much has been learned which is of use in diagnosis; this advance has been along the line of a better understanding, and a refinement and extension of medical diagnostic methods. What has thus far been done is but a beginning; much more may be expected from the continued application of this work in diagnosis.

ETIOLOGY

Much more important than these improvements in the art of diagnosis, are the fundamental advances in the science of etiology, initiated by colloid chemistry. It is difficult to outline this to the non-medical man. At most, we may hope to show from the historical development, what a profound change it has brought about in modern medicine.

Etiology is closely connected with the existing knowledge of natural phenomena. As with natural science itself, etiology also began with a physical era. From a medical standpoint, its most important contributions were the knowledge of the circulation of the blood and of the optics of the eye. To this was later added the results of chemical investigation, which brought an extremely great increase in knowledge, particularly of diseases of metabolism. The methodical application of chemistry (though in individual cases it went far beyond that) developed the knowledge of immune bodies with all its wonderfully valuable results, as a special and specific medical science.

But medical research had broken a new road for itself in still another direction; instead of the earlier humoral pathology, which explained all diseases on the basis of disturbances of the "humors," as a consequence of the development of microscopical technic there arose a cellular pathology, according to which the cell was regarded as an entity controlled by its own laws, whose independent behavior was etiologically determinative. Bacteriology, also the product of microscopic research, supplemented cellular pathology: disease was a struggle of cells against cells, the bacterium was the invading enemy, opposing which were the body cells as defenders. A microscopic picture of a section of tissue sometimes showed the morphological details of such a battle.

To the conception of disease above described, we could clearly apply the old principle with which Virchow once founded cellular pathology: "There-

* See paper by Dr. L. V. Heilbrunn in this volume. *J. A.*

fore if the matter is to be understood, and the details grasped, nothing remains but to go back to the elementary constituents; and the elementary constituents for the chemist are atoms, for the physicist molecules, and for the biologist cells."

Such a concept of etiology, however, separates the physician widely from the physicist and the chemist; the need of an understanding of the process is unsatisfied; pure vitalism dominates the details of disease as revealed by the microscope. The physician has long ago discovered the insufficiency of such a concept of disease. An attempt was made to explain the individual processes observed microscopically, by reference to simpler processes of physics or chemistry. These efforts yielded but slight results. The domains of physics and chemistry threw but little light upon the causes of the morphological changes observed in the body. Besides, the old so-called classical physical chemistry was incapable of helping the physician to make any extensive advance in etiology. Furthermore, as was the case with the attempt to apply physics and chemistry to body processes, it appeared that the principles of classical physical chemistry were likewise unable to explain what went on in the human body. Naturally the failure of these efforts was fuel to feed the flame of the vitalistic theory, and there developed an increased skepticism towards further efforts in this direction.

At this juncture scientific colloid chemistry was born. Even today, though colloid science has had but a decade and a half of development, there is unmistakably a revolutionary change of view in wide medical circles. Striking new results of far-reaching significance have led a not inconsiderable number of physicians to believe that in colloid research lies the key to profound penetration of the mysteries of etiology. Colloid chemistry has discovered the laws of the peculiar behavior of matter in microheterogeneous dispersion. Everywhere in the body, in cells, in intercellular tissue, in blood and in the body fluids are found colloids, some as sols of fluid nature, some as gels; practically every process bears the imprint of their individuality. Just for this reason in many cases which were previously incomprehensible to physics and chemistry, colloid chemistry was the magic wand to effect a solution. It was furthermore established that in practically every occurrence in the human body, it is indispensable to consider colloid chemistry, which has thus become a fundamental factor in etiology.

The vivification of medicine by colloid chemistry justifies us in calling this the beginning of a new era in etiology.

Today, in many fields, colloid chemical-medical research has already sowed the seeds for a rich harvest. It is impossible here to go into these matters exhaustively. Only in a few cases which are best suited to satisfactory description, will the attempt be made to give an insight into the nature of the new development.

Because of the extreme complexity characteristic of all colloid-biological problems, by force of necessity the first attempts to apply colloid chemistry involved the simplest possible conditions. Fluids extractable from the body cavities had the advantage that they could be investigated outside the body, and therefore free from all further cellular influence.

In such a manner, e.g., the question of the formation of gall stones and urinary calculi,* was attacked experimentally by colloid chemical methods. The nature of the origin of these concretions which had previously been com-

* See paper on Concretions, by H. Schade, this volume. *J. A.*

pletely unknown, is today greatly clarified (Schade). The principles underlying the peculiarities of structure and the conditions of formation of certain kinds of stones have been established; the critical factor for the formation of stones (as is described in detail by the writer elsewhere in this book), is the coöperation or interplay of crystalloids and colloids in precipitation and in guttulate separation.* This important example demonstrated the fruitfulness of colloid chemistry in etiology. Gall stones and urinary calculi, heretofore mysterious in origin because of their peculiar structure, have today become trustworthy documents, given us by Nature to enable us to trace the development of calculi-forming diseases.

Another field of greatest importance to etiology is the colloid chemical investigation of blood serum. Here viscometry first acquired importance. A most important factor in determining cardiac effort and the circulation of the blood, is the internal friction of the blood as a whole, that is of the blood plasma and the blood corpuscles together. Increase in the number of blood corpuscles or increased concentration of carbonic acid (CO_2), which latter causes great increase in corpuscular size as a result of swelling, may lead to a dangerous rise in the viscosity of the blood. Weakness of the heart, accumulation of carbonic acid, and increase in viscosity are for the physician a very significant trio, since they are often bound up in a "vicious circle." By slowing down the circulation, heart weakness brings about accumulation of carbonic acid; the accumulation of carbonic acid increases the viscosity of the blood, and this hyper-viscosity again throws more work on the already weakened heart. In pneumonia and arteriosclerosis the viscosity of the blood may be the critical factor for the outcome of the disease. If the danger is recognized in time by means of viscometry, resort to venesection, for example, may at one stroke avoid excessive blood pressure. This indicates one way in which colloid chemistry, by discovering a danger, may save life.**

Surface tension, as well as viscosity, may also disclose death-dealing blood conditions. If, as a consequence of a break in the vessels, substances such as air or fluid fat, which have high interfacial tension against blood, enter the blood stream, an embolism occurs at the adjoining capillary branches. The heart is not strong enough to bring about in these air or fat droplets a sufficient increase in surface to enable the deformed globules to pass through the small capillaries. The globules tend to remain in the forks of the vessels, blocking the lumen. Should such a blockage occur in the brain or in the lungs at some vital spot, death not infrequently occurs as a result of abnormally large interfacial tension.

The extreme consequences of viscosity and surface tension in disease have been intentionally mentioned here. Even the slightest deviations of these properties from normal most certainly have their significance in etiology. In the blood vessels, as in apparently all the hollow organs of the body, the colloid properties of the lining endothelial cells are such that the hollow organ is completely wet by its internal solution, i.e., between the two the surface tension approximates zero. The physiological consequence of the maintenance of a low surface tension at the walls of the hollow organs is that any precipitate which might perchance be formed in the fluid is not perma-

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* A realization of the importance of the influence of colloidal substances on crystallization and the formation of concretions, existed among a group of English physicians, whose work was far in advance of their time and was not appreciated. See "On the Influence of Colloids upon Crystalline Form and Cohesion, etc.," by William Miller Ord, M.D., London, 1879. *J. A.*
** See paper by Dr. F. P. Underhill in this volume. *J. A.*

nently adsorbed at the walls, so that the enveloping walls are always kept clean. As soon as the walls become diseased, all this is changed. Walls having cells of diseased colloid properties, immediately show evidence of adsorption; the wall covers itself with surface-active material from the fluid that laves it, and usually soon becomes "encrusted." In the blood stream these conditions most often cause thrombi adherent to the walls.

The various forms of colloid chemical research on the blood are today acquiring importance in the investigation of pathological questions. Testing of the stability of the blood colloids in various disease processes has especially come into prominence. The sedimentation speed of the blood corpuscles is often determined to give practical and theoretical information leading to a comprehension of diseased conditions.

Into the important field of the science of immune bodies, colloid chemical investigation has also made a successful entry: Ehrlich's theory has been shaken to its very roots by numerous and very specific colloid chemical discoveries, and everywhere we see a bursting of previously established bounds. Without doubt immunity will have a rebirth, but just what new form it will take is not discernible.*

Most striking results were obtained by colloid chemical medical research when it tested the readily obtainable body fluids, and took into account the behavior of the tissues themselves. (Schade and collaborators.) A large organ, connective tissue, weighing as much as 10 kilos in adults, was thus discovered, so to say, in so far as concerns its functional significance.

Connective tissue offered little promise for cellular pathological investigation, since it contains very few cells embedded in an extremely large amount of intercellular substance: it seemed, for the most part, to serve merely as a supporting and cementing substance, useful for mechanical purposes in the human body. It was reserved for colloid chemistry to discover, in this organ-system, functions of such general significance, that today connective tissue possesses great and incisive importance in etiology. It happens that the very same peculiarity which from the cellular pathological view had limited research, that is a paucity of cells in a highly developed intercellular substance, was, from the physico-chemical standpoint, of particularly favorable significance.

Connective tissue constitutes a great colloidal mass, disposed extracellularly, which on the one hand is inserted everywhere in the human body, between blood vessels and organ cells, like a many branched network, and on the other hand serves as a padding beneath the human skin, like a huge clinging mantle. For the physiology and pathology of the human body, considered from the standpoint of swelling, this mass of connective tissue has become a most important organ. The swelling of the connective tissue colloids in the human body is unsaturated, that is, the colloids have not reached the end point in their effort to absorb water.

The physiological swelling balance is determined by the following three factors: (1) an "equilibrium" (representing a normal) of the ions contained in the surrounding fluid (i.e., a faint alkalinity of pH = 7.45 and Na, K, and Ca in molar ratio of about 100:2:2); (2) the presence of a normal colloid-osmotic ("onkotic") protein pressure in the serum; and (3) the coöperation of a normal squeezing pressure due to the mechanical resiliency of the tissue itself. A bit of connective tissue removed from the body environment and

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* See paper by L. Reiner in this volume. *J. A.*

placed in a solution, usually swells far in excess of its ordinary physiological size. If, however, there are experimentally reproduced the trio of factors above mentioned, the extirpated pieces of connective tissue show exactly the same size as under normal body conditions. The swelling behavior in the human body, where normally this trio of factors hold sway, is accordingly basically the same as in the physico-chemical experiment.
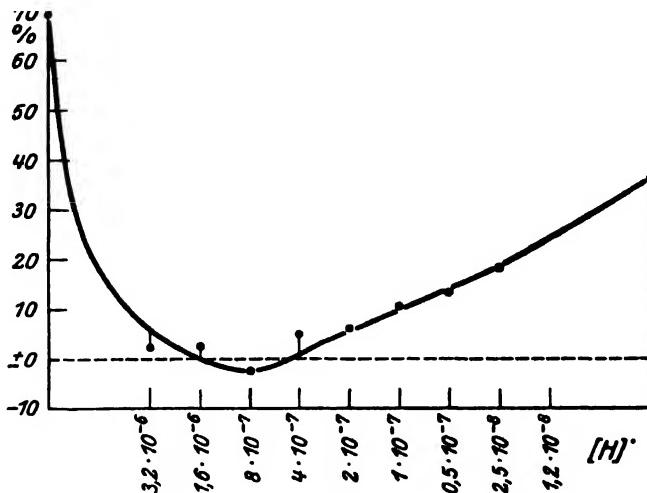
FIG. 1.—The Swelling of Connective Tissue as a Unit, within the Limits of $H-OH$-ion Concentration Occurring Intravitally.

Table 1 will give an idea of the extent of the variations in swelling, occurring as a consequence of pathological variations in the value of the factors above named.

TABLE 1. *Extent of the Effects Produced by the Factors Mainly Influencing the Swelling of Connective Tissue, in Terms of Variations in Swelling Brought About in the Course of 24 Hours.*

Factor Affecting Swelling	Variation in Swelling of Subcutaneous Connective Tissue		
(1) *Ions and Molecules:*			
(a) H — OH ion displacement occurring intravitally ((H) = 0 45 to 25.10^{-7})	about	15	per cent
(b) Variation of NaCl concentration between 0.6-1 2 per cent	"	3	" "
(c) Variation in concentration of urea from 0-300 mg. per 100 cc.	"	±0	" "
(2) *Colloids:*			
Variation in the protein concentration in the external solution between 4-8 per cent	about	15	per cent
(3) *Mechanical Pressure:*			
1 cm. Hg	about	10-20	per cent
3 cm. Hg	"	30	" "
6 cm. Hg	"	40	" "
3-4 cm. Hg = protein pressure of serum.			

The nature of the swelling of connective tissue with variations in H-OH-ion concentration is shown in Figure 1.

The behavior here figured applies to the connective tissue *in toto*. It is noteworthy, however, that the extracellular mass of the connective tissue is not a distinct entity, but is composed principally of two colloid-chemically antagonistic substances, the matrix or "ground substance" and the collagenous fibrils. Table 2 indicates this antagonism, based on the same main factors.

TABLE 2. *Antagonism in Swelling between Matrix and Collagenous Fibrils.*

	Matrix	Collagen
In pure water	Powerful swelling	Precipitation
With increasing NaCl concentration......	Swelling decreases	Swelling increases
In acid solution.........................	Slight swelling	Powerful swelling
In alkaline solution.....................	Powerful swelling	Slight swelling

To show in a single instance haw extensive this *antagonism* is, Figure 2 shows the behavior in acid-alkaline solutions.
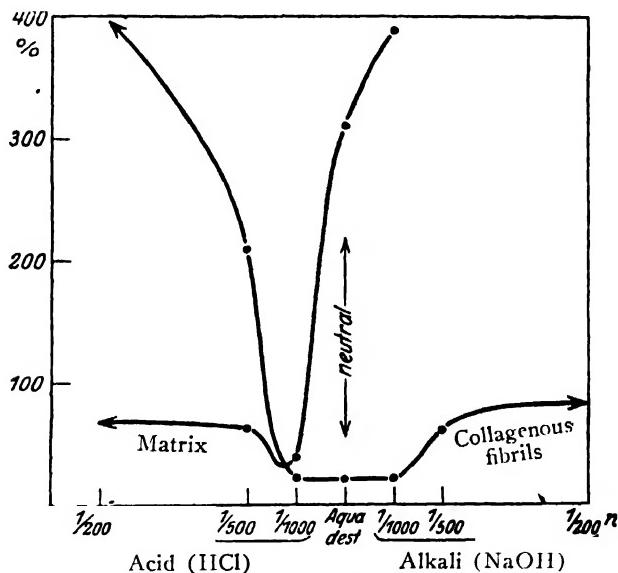
FIG. 2.—Swelling Antagonism between Matrix and Collagenous Fibrils in Acid-Alkaline Solutions.

There are in the human body tissues which consist practically only of one substance: the swelling of the umbilical cord embodies that of matrix, whereas sinew represents that of collagenous fibrils. Figure 3 shows the variations in swelling of umbilical cord matrix and of sinew (collagenous fibrils) in one of the experiments listed in the last table, in a range of H-ion concentration between 8.10^{-4} and 2.10^{-7}. This might be supposed to indicate a regularity generally applicable where two different colloids are subjected in the same environment, to a change affecting their swelling, were it not

that we have here the rare instance where both colloids have their swelling maximum at exactly the same points.

If the swelling minima are at different 11-OH-ion concentrations, there will always be found on transition from alkaline to acid reaction the three zones represented in Figure 3: first a zone of common shrinking, extending to a point where the first minimum is reached; then between this minimum and the next a zone of antagonistic swelling resulting in a more or less extensive stasis in the combined water of swelling; and finally beyond the second minimum a zone of renewed coördinated activity in the direction of combined swelling.

Connective tissue, under normal body conditions, belongs in the zone last mentioned; the efforts of both components, the matrix and the mass of collagenous fibrils, tend in the same direction. Under pathological conditions,
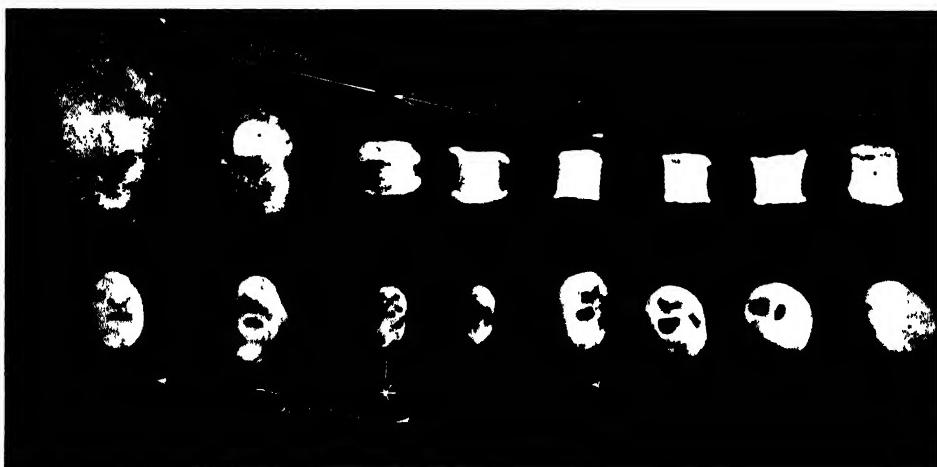
Fig. 3.—Tri-zoned Type of Coupled Swelling in the Case of Sinew and Umbilical Cord.

e.g., severe inflammation, however, connective tissue enters the zone of antagonistic swelling, and there comes into evidence (and this is important clinically), the principle of economization of water, wherein, as acidity increases, the water released by the matrix is immediately seized upon by adjoining collagen because of its swelling capacity, and wherein, furthermore, as the acidity diminishes, the water given up by the shrinking collagen is again taken up by the swelling matrix.

Such a retention must occur to a greater or less extent in all cases of "duplex swelling" as a consequence of the "three-zoned type." In the human body there are an usually large number of cases falling under this category.

Metabolism in the human body occurs in three principal domains, which are separated from each other by colloidal partitions. Figure 4 gives these relationships in schematic form.

The three great domains are blood, connective tissue, and cellular protoplasm. The capillary walls of the blood vessels and the "cell membrane" (which is present in at least a functional sense) serve as boundaries between these domains.

Because of its permeability to water, the capillary wall acts for the most part as a dialytic septum toward truly dissolved and colloidal substances. The cellular interface ("cell membrane"), on the other hand, in case of passive metabolism—in the process of cell nourishment "vitality" changes conditions—functions mainly like an osmotic membrane; that is, it is readily permeable to water (and also for lipoid-soluble substances, CO_2 and urea), but it is impermeable to other materials, especially to salt ions, the sugar molecule, etc. The division of the body into three parts by these two membranes is of highest importance for metabolic processes in both health and disease. Water finds no barrier anywhere in the body, and in spite of capillary walls
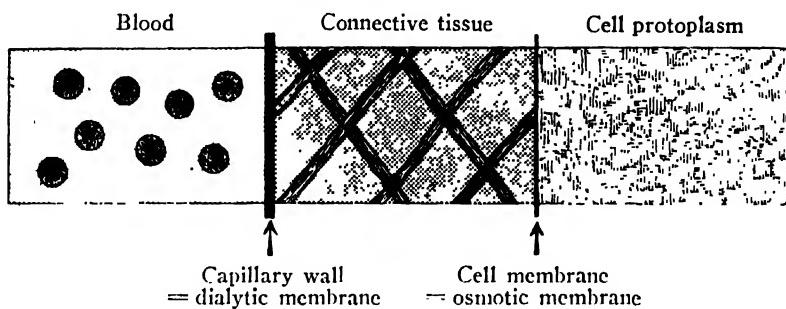
Fig. 4.

and cell membranes, can move to and fro among cellular protoplasm, connective tissue and blood. Crystalloidally dissolved substances are, on the other hand, mainly limited to the circuit connective tissue—blood in their simple physico-chemical interchange; the cell membrane is continuously permeable only for the two chief end products of metabolism, carbonic acid and urea (as well as for lipoid-soluble substances in general), and to them as to water, the entire domain of the body is available through diffusion.

For the interchange of colloids, the narrowest limits are established; they remain permanently confined to the domain in which they are found, be it blood, connective tissue or cell, unless perhaps by some special method a way is opened for their migration—e.g., by vital absorption on the part of the cell, or indirectly by means of decomposition into crystalloidally dissolved fragments, or the like.

The masses filling the three main domains themselves, are also of typically colloidal character, part sols as with blood serum, part jellies or gels as with connective tissue and cell protoplasm. The complexity of the colloid chemical phenomena in the case of connective tissue, has been briefly pointed out above in some particulars. The interplay of antagonistically acting colloid masses is, in the blood, just as important in controlling its internal auto-regulatory system. Here primarily red blood corpuscles and blood albumin act in direct antagonism. This is most obvious in the CO_2 transportation function of the blood, in continuous fluctuation of the CO_2 content in the course of the circulation between tissues and lungs. The slightest increase of CO_2 in the plasma makes the red corpuscles swell, while the slightest deviation toward the alkaline side makes their volume smaller. But the proteins in blood

show an exactly contrary behavior: upon the addition of the first small quantity of acid, whereby shrinking occurs as the isoelectric point and the swelling minimum are approached. On the other hand, with diminution of CO_2, that is with rising alkalinity, the albumin increases in swelling.

A similar antagonism also is shown in the action of salts; here the antagonism bewteen energy of swelling and osmotic energy is utilized in maintaining constant the total water content of the blood. The serum proteins taken as a whole, swell more with increasing NaCl concentration; the corpuscles, however, react osmotically, an increase of salt in the external solution reducing their volume.

An important rôle in maintaining constant the total water content of the blood, especially during its varying acidity, is furthermore played by a purely chemical factor, a high degree of buffer action; that is the blood proteins and blood salts are richly provided with "potential" ions. From the combined action of these factors there results an extremely far-reaching constancy over long periods of time, of the water held in the blood; and this constancy is a fundamental factor in stabilizing the swelling behavior of the neighboring domains, connective tissue and cell protoplasm.

Only recently has it been possible to measure the colloid-osmotic pressure ("onkotic pressure") of blood plasma by means of a new, rapid and at the same time accurate method (onkometry of fluids according to H. Schade and P. Claussen). The onkotic pressure of the blood plasma, during health, maintains itself constant within the narrow limits of about 2.1 to 2.9 cm. Hg. This equilibrium of the swelling pressure of the blood is of the greatest importance in the swelling-physiology and swelling-pathology of the human body. It forms everywhere throughout the body a stable and normal level, to which the swelling pressure of the connective tissue and furthermore the organ cells can conform.

After the lungs have, by way of preparation, exactly regulated the H-ion concentration of the blood, the kidneys, in the further course of circulation, by eliminating a certain amount of the water of solution, strives to reach anew the narrow zone of swelling pressure, to which the other colloids of the body, according to their hyper- or hypo-pressure, may put themselves in constant balance, either by giving up water or absorbing it.

We have as yet very little intelligible knowledge regarding the colloid behavior of the third domain, the protoplasm in the living cells themselves. This much is certain, that the condition here, as compared with the area of the blood and the extra-cellular mass of connective tissue, are considerably more complicated. At present we may state that there are colloidal antagonisms in the cell protoplasm, principally between the nucleus and the protoplasm, further between the different kinds of granules of the protoplasm, also between the proteins and the lipoids, and finally also between the cellular albumins, which differ chemically among themselves.

For the present, therefore, the cell constitutes an inextricable mosaic of substances of the most diverse colloid properties. Meanwhile there has been considerably better clarification of the swelling relationship between the swelling of the protoplasmic mosaic as a whole, and that of the surrounding connective tissues. We now know that, insofar as concerns swelling, there is an important antagonism between connective-tissue colloids and cells in their water requirement. This is shown by the following table:

TABLE 3. *Swelling Antagonism between Connective Tissue and Cell.*

	Acid	Displacement of the Level Toward		
		Alkali	Hypertonicity	Hypotonicity
Connective tissue	—	+	+	—
Cell	+	—	—	+

+ = swelling   — = shrinking

Physiologically such opposition must be very effective in establishing the water balance of the cell. Competition for requirements is avoided as far as possible. If surrounding conditions are such that the cells need water to swell, it is offered them voluntarily from the connective-tissue area; and on the other hand in case the cells give up water by shrinking as the consequence of a change in the surrounding medium, this water is fixed by the connective tissue, whose swelling increases. Coincident with the ease of water interchange resulting from this arrangement, goes the principle of economy of water requirements. This is so because excess water of the cells is immediately fixed by swelling of the adjacent connective tissue, so that later when conditions change, water is available again for the protoplasm.

Because of this antagonistic adaptation, connective tissue, colloid, and organ cell, are especially well suited to symbiosis. Normal functioning of human cells is possible only if we have normal connective tissue. Morbid changes in the colloid condition of the connective-tissue area must lead to injury of the cell itself.

The phenomenon of swelling in the human body has still another peculiarity of colloid chemical interest: the adjustment of swelling almost always occurs between systems that are kept in motion mechanically. The heart is the motor which keeps the colloidal fluid of one system, the blood, in constant circulation, and, thanks to the simultaneous coöperation of the kidneys and the lungs, always forces it through the capillary system of the tissues at a constant level of concentration.

A very remarkable phenomenon appears when a colloid solution, under slight hydrodynamic pressure, flows through a tube whose walls possess marked dialytic permeability. Whereas, during the circulation of a colloid-free solution, water is forced through the dialytic wall along the entire length of the tube as long as there is still within it a positive pressure, there is a basic difference when a colloid-containing solution is circulated. In the latter case the passage of water through the dialytic vessel walls occurs only up to that point of the tube where the mechanical pressure forcing the water out has sunk so low that it is exactly balanced by the onkotic pressure of the colloids in solution there. From that point onward, as the mechanical pressure continues to fall, the onkotic pressure gains more and more the ascendency, and accordingly water is drawn in from the outside through the dialytic wall (Schade and Claussen).

As we know, in only a portion of its length, the capillaries, is our circulatory system provided with a thoroughly permeable wall for dialysis; which means that only here is it possible for there to be any considerable interchange of substances with the tissues. It is quite characteristic that in all capillaries this section open to dialytic interchange is so located, that the balance between the mechanical and the onkotic pressure is found exactly in the middle of the

section, where therefore also occurs the transition point between the dialytic outward and inward currents.

Therefore from the first sections of the capillaries, water containing true solutes is forced by the predominant mechanical pressure. Then follows the transition point in the dialytic stream, and in the further sections of the capillaries because of the predominance of the onkotic pressure there, water containing true solutes is drawn into the blood. This interplay between hydrodynamic pressure and onkotic pressure is the main and basic cause of the constant para-capillary flow of lymph in the connective-tissue area. Colloid energy appears, within these narrowly defined limits of the capillary, as the driving force of the flow of fluid from tissue to blood. This method of utilizing the colloid energy in connection with the variation of the hydrodynamic energy of the blood stream, is of the greatest importance for the fluctuation in the appearance of capillary transudation and resorption.

Thanks to colloid chemical knowledge of adsorption, we now have an increased breadth of the medical view regarding the behavior of tissues as depots, which is analogous to that briefly outlined above for the physiology and pathology. We refer here to the deposition and liberation, in health and in disease, of substances involved in the metabolism of protein, carbohydrate and fat, as well as mineral substances. It would take too much space to detail the material already developed by this new research. The general applicability of the process of adsorption to the problem referred to is obvious to the colloid chemist.

This research is fraught with great medical significance in still another direction; namely, the part played by the blood and connective-tissue colloids in regulating certain general constants essential to the maintenance of life—osmotic isotonicity and H-OH-ion concentration.

Clinical experience indicates that even without the coöperation of the regulatory organs of elimination (kidneys, lungs, etc.), these constants may be maintained for quite some time with astonishing regularity. This is the result of the so-called "inner regulation." A broad point of view is needed here. The purpose of isotonicity and isoionicity is to protect the cells of the body from injury as far as possible, by giving them a practically uniform optimum environment. Since this is the purpose of the constancy of the blood, in carrying on the balancing of substances continuously necessary to maintenance of these constants, the body cannot use the very cells it is desired to protect. The regulation of the body fluids must be effected in some third place where there are neither cells nor blood. That place is the extracellular colloid mass of the connective tissue.

Analysis shows that the balance actually is effected this way. Medically speaking, the first great danger to the blood constants lies in acidosis. To meet this condition the connective tissue has in particular the collagenous fibrils, which are highly acidophilic substances and according to the laws of adsorption act as specific "acid-catchers," thus greatly aiding the maintenance of equilibrium. The second great danger to the blood constants is the possibility of an osmotic excess. Here again a common colloid phenomenon acts in a regulatory fashion: the connective-tissue matrix belongs to that class of colloids which shrink when the salt-content of the surrounding medium increases. If there is danger of too high a salt concentration in the blood or the tissue juices, the matrix by shrinking supplies solution water for dilution.

These and other similar processes made possible by the antagonism be-

tween swelling and osmosis and the variations in swelling of the individual colloids, the proteins, and particularly the colloid mass comprising the connective tissue, are enabled to play an active part in the regulation of the constants of the body, both in health and in disease.

A number of body processes by no means inconsiderable in range and significance, have been freed from the domain of "vital processes" and brought nearer to, or actually led to exact explanation by the colloid chemistry of the blood, the tissue juices, and the extra-cellular colloids of the connective tissue. In extensive fields heretofore approachable, if at all, only morphologically, we now see mechanical, osmotic and colloid forces coöperating in service to the body as a whole. An entirely new light has been thrown on the question of water economy by our knowledge of the physiology of swelling, and on the question of the transfer of salts and nutritive substances by our knowledge of adsorption. These newly developed ideas have facilitated the solution of many important clinical problems. To show this a few chosen instances must suffice.

ORIGIN OF EDEMA

This question was early attacked by M. H. Fischer, but with the then imperfect methods. It is now so well understood that today we distinguish by their physico-chemical characteristics, four clearly defined kinds of edema:

(a) *Swelling-edema, consequent on abnormal increase of swelling capacity* of the tissue involved. Here belong alkali-edema and among the salt-edemas, those caused by iodine compounds in particular. The hypothesis that swelling edemas are caused by acids has proven untenable.

(b) *Swelling-edema consequent on a drop in the swelling pressure of the blood.* Here belong primarily edemas of kidney diseases, where the kidney loses the power of reducing the water in the blood to a normal pressure value, so that the tissues in establishing their onkotic balance receive more water from the blood. Of further importance with nephritics is the modification of conditions in the mechanics of flow. Because of lowered onkotic pressure of the blood the transition point from outward to inward dialysis (*vide supra*) is forced backwards along the capillaries, so that the outflow from the blood to the tissues exceeds the reverse current. This practically amounts to pumping fluid from the blood to tissues which then assume their swelling equilibrium as before pointed out.

(c) *Edema mainly of osmotic origin.* The edema of inflammation represents this type. Here osmotic hypertonicity on the part of the tissue so dominates the sum total of forces that osmosis practically controls the motion of fluid. Another contributing cause is the greater hydration capacity of abnormal proteins formed in the tissue by chemical changes in the course of inflammation.

(d) *Edema caused mechanically.* To this group belong the congestive edemas due to interference with the circulation. Here the criterion is increase in the mechanical pressure of the blood in the venous section of the capillaries. When the increase in the mechanical pressure within the capillary extends as far as the venous portion, both the zone and intensity of the outward dialytic current are increased, while the return current from tissue to blood is diminished. Consequently fluid accumulates in the tissue.

These various ways in which clinical edema may arise are particularly mentioned here, because in the technical colloid chemical literature edema is frequently considered from a single standpoint.

of the individual body cells. During the cellular pathologic era this concept could hardly hold its own, much less make progress. Microscopical morphology was unable to find in the appearance of the cells any differences corresponding to marked differences in constitution.

Colloid chemical medical research has been able to make material progress in this problem. As we have pointed out above, cells and connective-tissue colloids exist in close symbiosis. To the cells of the human body, as well as to plants, there applies the old rule that growth and welfare are closely connected with the properties of the "soil."

But, however, the properties of this "soil," that is, the connective-tissue colloids, differ remarkably in the various individual types of human constitution.* The most important variation from normal are the delicate, the heavy set, the flabby (mechanically speaking), and the puffy, having thickened connective tissue, which is swollen, in a mucous-like fashion, by depositions of material. To each one of these deviations there regularly corresponds a certain pathological behavior of the body as a whole, and corresponding to the series just given, we have the nervous, the phlegmatic, the asthenic, and the myxedematous constitution.

The nerves, which are the most sensitive part of the organism, reflect with especial clearness, the characteristics impressed upon the organ cells by the connective-tissue colloids: the abnormally tender "soil" corresponds to an abnormal speed of nervous reaction, the heavy set "soil" to an abnormally slow nervous reaction, the asthenic "soil" to weak and irritable nerves, and finally the myxedematous "soil" to the most extreme limitations of the nervous system, even to the limit of idiocy by the negation of all functions. The colloid structure of the connective tissue is thus shown to bear the closest relation to cell or organ functions.

Cells can function normally only in a healthy connective tissue. If the colloid-physical or colloid-chemical properties of the connective tissue depart materially from the normal, so that, for example, there arise disturbances in water transfer, diffusion, or adsorption limits, this makes itself felt throughout the whole range of the cells affected. The discovery of this colloidally influenced relation between connective tissue and cells has opened to exact experimental investigation a field of quite general significance for etiology. This increased range of medical knowledge has carried the physician's ideas beyond the limits set by cellular pathology. Neither the cells alone, nor their groups which form organs, constitute the entire body, but in addition there are the great masses of blood fluid, the tissue juices, and the extracellular connective-tissue colloids, which are active in the most important problems.

By far the most important consequence of the vitalization of etiology by colloid chemistry lies perhaps in the fact that the perception of disease processes can be removed from the limitations of cell and organ specialism, and by being once more brought into relation with the old humoral pathology, reach a freer mean position, where account can be taken of the body juices and the extra-cellular tissue colloids as well as of the cells.

THERAPY

Therapy has beyond doubt, up to the present, especially insofar as concerns internal medicine, labored under a considerable and basic limitation due

* Galen recognized the following temperaments: (1) sanguine, (2) phlegmatic. (3) bilious or choleric, (4) melancholic And medical men used to speak of diathesis, generally meaning predisposition to some disease. *J. A.*

to cellular pathology. So long as the cells alone are regarded as being responsible for disease, that is, if the disease consists merely in the alteration of a vital process, then therapy consists only in helping the cells get well again. The main methods of gaining control over the course of a disease would be by rest or exercise, by excitation or paralysis, by increasing cell nourishment, or by killing invading bacteria. It was unavoidable that the mortality rate remained unsatisfactory. Consequently great skepticism arose. The heyday of cellular pathology coincided with the time when the therapy of internal diseases tended to be regarded as worthless.

The results of the clinical treatment of tuberculosis since the time of Brenner, the results of light treatment since the time of Finsen, and the results of treatment with other kinds of rays enabled medical practice to break this ban; but these great practical advances were not accompanied by an incidental increase in the theoretical knowledge. Today, however, colloid science has paved the way for a basic advance in our knowledge of therapy, and this advance is along two lines: first it has shown that the eucolloidality (optimum colloidal state) of the protoplasm is a prerequisite of normal cell function; and second, apart from the cellular field, it has shown the importance of the extra-cellular tissue colloids, which has opened up a great field, rich in the possibilities of colloidal influence.

Many medicaments will be used in colloidal form as human remedies. Modern colloid technic has already produced about a hundred new preparations for therapeutic use. Among the most highly regarded are especially metallic Ag, Hg, Fe, and also S and Ca salts. The advantage in using substances in colloidal state is that, in those water soluble, the enormously increased surface leads to intensive action. Medically, the use of water-soluble colloids is indicated, wherever the substance in question, if in ionic dispersion, would immediately react with the substances it would meet in the body, or when action is desired at a remote place, or when the action is to be spread over an extended period.

Colloid chemical experience also plays a part in deciding how drugs should be administered. Not infrequently the sequence of administration is of considerable importance: thus injections of strophanthin should not be given if the heart has just previously been treated with digitalis. Not a few remedies, in their action, materially depend on the ionic constitution of the milieu. Not only may the influence of various ions materially reduce or even prevent the therapeutic action of a remedy, but it may even reverse the normal action. Two remedies given simultaneously usually do not give a simple combined effect, but frequently an "intensification"; so that recently, instead of using pure substances, many prefer to use artificially made combinations, or else revert to the use of vegetable drugs where the medicaments are found in natural combination.

It is not difficult to find colloid chemical relations throughout the whole field of therapy; or, putting it the other way about, it is difficult to find remedies to which such considerations do not apply. The reason for this is quite simple. Every expression of cellular life is associated with a colloidal substrate. Anything that is to exert a therapeutic action on the cells or the tissues of the human body, must therefore, be able to come into direct or indirect relation with colloids. A therapeutic method which would in no wise reach the colloids of the body would consequently not be worthy of the name.

Numerous therapeutic procedures aim their effects directly at the cell colloids. Substances which dissolve colloids are used as "solvents," e.g., alkaline salts to fluidify mucus in catarrh. In catarrhal conditions of the eyes, nose, stomach, intestines, and urinary tract, salts of aluminum, silver, copper, zinc, bismuth, and lead are used as "astringents" to precipitate mucus and albumin. Ferric chloride and calcium salts are used as "styptics," to stanch the flow of blood. As superficial caustics, there are used acids or salts, which like nitric acid or chromium salts, for example, make a "cautery boundary" of the "precipitated" albumin. For deep cautery there are used agents which, like potassium salts, reduce the tissue albumin and the horny substance of the skin to a semi-fluid condition, so that the preliminary cautery layer is no bar to the deeper penetration of the agents.

If the tissues are too permeable to diffusion, calcium is often of use; on the other hand if the tissues are hardened, as in the aged, or have a lowered permeability to diffusion or lowered colloid-physical or colloid chemical properties, iodides are very useful in aiding the tissues toward the sol state.

Cardiac stimulants exert part of their effect on the colloids of the heart muscle. The proportionate concentration of veratrin in the fluids of the heart sac, is an outstanding instance of the application of Freundlich's adsorption law in biology. Diuretics also exert a powerful influence on the swelling behavior of the body colloids. The therapy of activating protoplasm by protein injection or other procedure goes deep into problems of colloid chemical nature. Practically all the problems involved in immune body therapy are closely connected with colloid science. The same is the case with the ferment therapy of the stomach and intestines.

The whole science of dietetics with its problems of the preparation and digestion of foods, also belongs as much to colloid chemistry as to medicine. Adsorption therapy; with the aid of charcoal, bolus alba, and other finely divided substances owes its scientific basis and its development almost entirely to colloid chemistry. Questions involved in the external and internal disinfection of the body cannot be solved without the aid of colloid chemistry. Narcosis is attained by a variety of methods, having as a common object a colloidal change of the protoplasm: by lipoid-soluble substances which precipitate protoplasm proteins or render them inactive by envelopment in a lipoid film, by protein precipitations in the milieu, and finally by lowering the temperature below the limit necessary for protoplasmic eucolloidality. General narcosis with chloroform, local anesthesia by injection of salt-free water, and "freezing" anesthesia with ethyl chloride on the skin, are examples of each group.

Balneology with its problems of the effects produced by salts in mineral waters, is of colloid chemical interest. The basis of all activity in this case is the physical and chemical interchange between the ions brought in by the mineral water, and those in the body colloids. In fact the existence of osmotic isotonicity and H-OH as well as Na-K-Ca-isotonicity offers a very considerable resistance to efforts to alter the constitution of the body juices in a therapeutic sense. But as Jung's metabolism experiments show, there are not excluded small changes of practical significance. The mucous coat of the stomach and intestines, as well as the mucous coat of the respiratory and urinary tracts, are more readily reached by therapeutic ionic influence. These tissues are therefore the main ones open to mineral water therapy. When disease has brought the mucous membrane cells to a changed colloid state,

the therapeutic ideal, from a colloid chemical standpoint, is to cause a retrogression as far as possible to the original state of colloidality.

Inflammation, by far the most common form of disease, is accompanied by colloid changes among which swelling of the cell colloids is the most important. In all catarrhs the mucous membrane cells are abnormally swollen. As may be shown, it is possible by the use of mineral waters of proper constitution, to overbalance the colloid solvent ions by those of precipitating nature, that the desired colloid chemical correction is brought about in the cells.

Antionic Therapy. The ancient concept of "anti-catarrhal action," which was denied by cellular pathology and therefore quite forgotten, has, by the application of colloid chemistry, been given precise limits, and has enriched therapeutics with a method of cure which rests on a new principle. This result in so important a field beautifully illustrates the quickening influence of colloid chemistry for practical therapy.

We have here shown the intimate relation between colloid chemistry and internal medicine. To bring detailed proofs of this is not possible within the limits of this paper. Many of the results are taken from the work of the author and his co-workers. Those who desire a detailed and comprehensive discussion of this field are referred to H. Schade's book, "Physical Chemistry in Internal Medicine," now in its third edition (Th. Steinkopff, Dresden).

The Modifications of the Dynamic Surface Tension of Plasma and Serum

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I. INTRODUCTION

Surface tension is the force which tends to render the free surface of a liquid as small as possible. A distinction is made between the dynamic and the static surface tension.[1] These two values are generally identical for pure liquids (e.g., water). They also do not differ at all for molecularly dispersed solutions (e.g., dilute acetic acid). But this is not the case for complex biological fluid media containing colloids.[2]

To determine the surface tension of a solution, there are numerous methods based upon different principles. In some of these methods the dynamic, while in others the superficial surface tension is measured.

During recent years there have been often employed in biology methods based upon the number of drops of fluid given by Traube's stalagmometer[3] or by Kopaczewski's tonometer.[4] The dynamic surface tension is thus obtained.

Lecomte de Noüy[5] on one hand, Brinkman and Miss E. van Dam[6] on the other hand, proposed to measure the static surface tension by determining the force necessary to tear off a liquid film adhering to a ring pulled out of the liquid under examination.

According to Freundlich[7] and Lecomte du Noüy it is very difficult to determine the surface tension with a great accuracy. Even for pure fluids it is necessary to make allowance for an error of at least 1-2 per cent according to Freundlich. An agreement is still very remote with regard to the surface tension of water at its contact with air. Kalähne and Freundlich[8] collected the values for water at 18° C. as given by different investigators. They range between 72.3-76.8, the most probable value being 73 dynes per cm. The error is certainly considerably higher for body fluids which are of very complicated chemical composition. It can therefore be easily understood that the values obtained up to present by different authors for the same biological

[1] Freundlich, H., "Kapillarchemie," Leipzig, p. 7 et seq., p. 69 et seq., **1923**.
[2] Tommaga, T., "Über die Bestimmung der Oberflachenspannung biologischer Flusigkeiten mit der Torsionswage," *Biochem. Z.*, **140**, 230-253 (1923).
[3] Traube, J., "Physikalischechemische Methoden," Hamburg u. Leipzig, 42-46, **1893**; Traube, J., and Blumenthal, F., "Der Oberflachendruck und seine Bedeutung in der klinischen Medizin," *Z. exper. Pathol. Therap.*, **2**, 117-132 (1905).
[4] Kopaczewski, W., "La tension superficielle et sa mesure; un nouveau tonomètre," *Arch. phys. biol.*, **1**, No. 4, 23 (1921).
[5] Lecomte du Nouy, P., "A new apparatus for measuring surface tension," *J. gen. physiol.*, **1**, 521-524 (1919).
[6] Brinkman, R., and van Dam, Melle E., "Eine einfache und schnelle Methode zur Bestimmung der Oberflachenspannung von sehr geringen Flussigkeitsmengen," *Münch. med. Wochenschr.*, **60**, 1550-1551 (1921).
[7] Freundlich, H., *loc. cit.*, **34**.
[8] Freundlich, *loc. cit.*, **33**.

medium show great variations even when determined by the same method. It is therefore advisable to be rather careful in their interpretation as Lecomte du Noüy [9] rightly pointed it out in 1923.

It seems, however, that by observing all necessary precautions it is possible to determine the surface tension of serum and particularly of plasma with a degree of accuracy, relative, no doubt, but still permitting the detection of changes in these media under certain given conditions.

This appeared to us more difficult with regard to the static surface tension, at least when employing the torsion balance. Kopaczewski,[10] too, made some proper critical remarks with regard to this problem. Lecomte de Noüy's [11] recent improvements in the technique of the determination of static surface tension make possible a series of determinations, if there are only enough specimens of the same medium at disposal. But the errors caused by evaporation still remain, even though he uses an appropriate but relatively complicated holding device.

The study of the coagulation of blood plasma and the methods based upon tearing away a ring permit, to a certain extent, an estimation of the changes in surface tension of a medium whose physical state gradually changes.

Be this as it may, we will here discuss only the principal facts, which we were able to prove during recent years, regarding the surface tension of plasma and of blood serum examined under different experimental morbid conditions and we will subsequently analyze the results in connection with the static surface tension of these media.

II. Method Employed to Measure the Dynamic Surface Tension of Plasma and of Serum

Traube's stalagmometric method, while simple and of a convenient technique, has certain serious inconveniences (the difficulties in refilling; large amount of fluid required) and neglects some important factors.

It is particularly unfortunate, that the method does not take into account the evaporation of the fluid under examination, a point regarded as very important by Kopaczewski [12]: the drops form, in fact, in air, and this causes considerable error, particularly when dealing with volatile substances, like alcohol.

Traube enlarged the inferior extremity of the capillary tube considerably in order to increase the surface from which the drops are detached, so as to avoid the fluid creeping up along the exterior edge of the opening. Consequently no drop is ever completely detached, but a certain amount of fluid always remains adherent to the detachment surface. This quantity depends upon the adherence area and upon the surface tension, as Traube pointed out.

Kopaczewski's tonometer avoids certain of these inconveniences. We employed also this method for measuring the dynamic surface tension of plasma and serum.

Guye and Perrott [13] have clearly proved that it is necessary to count the

[9] Lecomte du Nouy, P., Les phénomènes de tension superficielle en biologie," *Compt. rend. Soc. Biol.*, **89**, 1076-1077 (1923).

[10] Kopaczewski, W., "Tension superficielle en biologie," *Compt. rend. Soc. Biol.*, **91**, 402-404 (1924).

[11] Lecomte du Nouy, P., Surface tension of serum, XI. An improvement on the technique for measuring surface tension." *J. gen. physiol.*, **6**, 625-628 (1924).

[12] Kopaczewski, W., "Théorie et pratique des colloïdes en biologie et en médecine," Paris, 98-116 (1923).

[13] Guye and Perrott, *Archives des Sciences physiques et naturelles,"* **11**, 4° série, 1, 1901 and 15, 132 (1903). See Kopaczewski, *loc. cit.*

number of drops and also the time necessary to form the drop, in order to calculate the surface tension from the weight of a drop suspended from a free surface of detachment. Following Kopaczewski's [12] advice we have used the classical formula of Tate,[14] with Guye's and Perrott's correction.

$$\alpha = K \frac{d_1}{N_1} (1 + \beta) + \gamma.$$

In this formula α represents the surface tension; K a constant which varies with the standard liquid of known surface tension, d the density of the standard liquid (water, alcohol, etc.) ; N the number of drops of the standard liquid; d_1 the density of the liquid being tested; N_1 the number of drops of the liquid tested; β the ratio $\frac{N_1}{N}$ of the number of drops of the liquid tested to the standard liquid; and γ the relation $\frac{T_1}{T}$ of the time of the formation of a drop of the liquid tested to the time of the formation of a drop of the standard liquid.

We checked the accuracy of the tonometer, by counting the number of drops N of distilled water before and after each series of determinations.

We carried out part of our research by placing the tonometer in a thermostat set at 18° C. Unfortunately we did not do it in all cases; sometimes we made our determinations at room temperature (at 18-20° C.), carefully registering with a precision thermometer the temperature inside of the container, which surrounded the tube, from which the drops escaped. In order to be able to use Guye and Perrott's formula, we furthermore measured the number of seconds required for the outflow of the standard liquid (distilled water), and of the plasma or serum being tested.

Beginning with our first experiments we calculated the surface tension of the medium under test, by accepting Landolt-Börnstein's value [15] 73.26 dynes, as the surface tension of water at 15° C. It seemed to us afterwards more desirable to accept Freundlich's authoritative opinion, and adopt 73 dynes as surface tension of water at 18° C. The results obtained by the two methods do not differ generally more than by $\frac{1}{10}$-$\frac{2}{10}$ of a dyne per centimeter for the media tested.

III. Dynamic Surface Tension of Plasma in Different Mammals and in Man

Cosmovici [16] deserves credit for establishing in 1915 the fact that in vertebrates the surface tension of the plasma is higher than the surface tension of water and particularly higher than that of serum obtained from the same blood, which latter is lower than the surface tension of water.

Cosmovici's method is without doubt not entirely above criticism and subsequent researches have not completely confirmed the numerous data published by this author. Nevertheless, by using the Kopaczewski [4] method and by calculating the dynamic surface tension at 18° C. with the corrections according to Tate and Guye and Perrott, it is easy to verify that normal mammalian

[14] Tate, *Phil. Mag.* (4), **27**, 176 (1868). See Kopaczewski, *loc. cit.*
[15] Landolt-Börnstein, "Physikalisch-chemische Tabellen." 3rd ed., Berlin, 102, 1905.
[16] Cosmovici, N. L., "La tension superficielle du plasma et du sérum sanguin avec applications à l'étude de la coagulation du sang," *Ann. scient. Univ.*, Jassy, **9**, 365-462 (1915).

plasma has a higher surface tension than water and the serum a lower surface tension than water.

Take for this purpose animals (dogs, guinea pigs, rabbits) which have been starved since the previous day. We introduce a paraffinized cannula into the carotid. Discard the first drops of blood, because they might be contaminated with tissue juice. Collect 9 cc. of blood directly into a centrifuge tube containing 1 cc. of an anticoagulating fluid. Mix it well with the blood, avoiding a liberation of hemoglobin. Centrifuge for 2 hours at a high speed, pipette off the plasma and centrifuge a second time for 20-30 minutes, in order completely to get rid of all cellular elements. Then immediately proceed to determine the density and the surface tension of the clear plasma thus prepared.

We used the following anticoagulating mixtures:

1. *Oxalate solution:* NaCl, 0.65 gr.; sodium oxalate, 1 gram; double-distilled water, 100 cc.

2. *Citrate solution:* Sodium citrate, 3.55 grms.; double-distilled water, 100 cc.

3. *Hirudin solution:* 2 mgr. hirudine per cc. of aqueous 0.85% NaCl.

While it is relatively easy to obtain a perfect plasma from animals by the method described, this is not the case with man. In fact, it is necessary to introduce in this case a needle of suitable caliber through the preliminary disinfected skin into a vein at the bend of the elbow. It is absolutely necessary to avoid contamination with tissue juice and to suck up the blood quickly, being sure to mix it well with the anticoagulating solution, but cautiously so as to avoid liberating the hemoglobin, which might happen if shaking is too vigorous, because some erythrocytes are then broken up.

If it contains hemoglobin, it must be rejected. In fact the coloring material of the blood does greatly diminish the surface tension of water. If hemoglobin is dissolved in plasma in the proportion of 1 : 1000, the surface tension is considerably lowered (12-14 dynes per cc.).

It is also important not to take a specimen of blood after a meal rich in fat. The plasma is then more or less cloudy, due to small fat droplets and the surface tension is then smaller, than in a specimen of blood taken from the same person after fasting, or after a light meal devoid of fatty materials. The difference between the observed figures in the two conditions may reach 2-3 dynes per cc.

Two specimens of blood taken from the same person at an interval of 30-60 minutes after fasting, do not show any appreciable difference in surface tension. The same is observed when the two blood specimens are taken after a light meal devoid of fat.

In order to determine the normal value of surface tension in man, the specimen of blood was taken in the morning before breakfast or after a very light meal without fat or with very little fat.

Table I shows the minimum, maximum and average surface tension, obtained in a number of experiments with dogs, guinea pigs, rabbits and man. It clearly shows how small are the variations of surface tension of plasma in a normal state: 1.6-2.7 dynes per cc. at 18° C. for the different individuals of the same species and the same kind of plasma. The minimum, maximum and average values differ but little, according to the nature of plasma or the species of animals. The constancy of the surface tension of plasma for the same species can be verified in man.

TABLE I.

		Surface Tension of Plasma in Dynes per cc. at 18° C.		
		Minimum	Maximum	Medium
Dog	oxalated	74.6	76.7	75.8
	citrated	74.8	76.9	75.8
	hirudin	75.2	76.7	76.0
Guinea pig	oxalated	74.2	76.6	75.7
	citrated	74.1	76.8	75.7
	hirudin	74.4	76.7	75.5
Rabbit	oxalated	74.4	76.5	75.4
Man	oxalated	74.1	76.7	75.2
	citrated	74.0	76.4	75.0
	hirudin	74.4	76.2	75.1

Let us now consider both sexes separately. The surface tension varies in the male between 74.1 and 76.5, or corresponds at an average to 75.1 dynes per cm. In the female it is between 74.3 and 76.7, averaging 75.4 dynes per cm. The dynamic surface tension of oxalated plasma seems to be somewhat higher in women than in men, although the number of cases studied is at present too small to draw a definite conclusion in this matter.

According to Brinkman,[17] the static surface tension of oxalated plasma is from 55.4 to 57.2 in men, and from 59.2 to 61.5 dynes per cm. in women. Here the difference is marked and constant. Brinkman refers this to the greater rapidity of sedimentation of the blood cells in women, pointed out by Fåhraeus [18] and confirmed by Plaut,[19] Runge,[20] Benninghof,[21] Büscher [22] and numerous clinicians. There is no evidence of a distinct influence of age upon the surface tension of plasma.

The nature of the anticoagulant added does not seem to have any influence upon the surface tension of plasma. In fact, on taking three successive samples of blood from the carotid of a dog, and preparing them with oxalate, citrate and hirudin as above described, we find but slight differences in their surface tension. See Table II for several examples of this kind.

The quantity of blood obtainable from a guinea pig is not sufficient to yield several samples from the same animal. But by using several guinea pigs it is possible to make mixed plasmas, each containing the same proportions of different blood samples. Thus two guinea-pigs can each give 10 cc. of blood, half of each specimen being oxalated, the other half hirudinized; and the respective halves are mixed. On determining the surface tensions of

[17] Brinkman, R., "Sur la tension superficielle du sang humain normal," Arch. néerl. Physiol., 7, 258-262 (1922).
[18] Fåhraeus, R., "Uber die Ursachen der verminderten Suspensionsstabilität der Blutkörperchen während der Schwangerschaft," Biochem. Z., 89, 355-364 (1918). "The suspension stability of the blood." Acta medica scandinavica, 55, 1-28 (1921).
[19] Plaut, F., "Untersuchungen über die Senkungsgeschwindigkeit," Münch. med. Wochenschr., 57, 279-282 (1920).
[20] Runge, W., "Über die Senkungsgeschwindigkeit der roten Blutkörperchen bei Gezunden und Geisteskranken," Münch. med. Wochenschr., 67, 953-955 (1920).
[21] Benninghof, F., "Klinische Untersuchungen über die Senkungsgeschwindigkeit der roten Blutkörperchen im Citratblut," Münch. med. Wochenschr., 68, 1319-1320 (1921).
[22] Buscher, J., "Zur Frage der Senkungsbeschleunigung der roten Blutkörperchen," Berl. klin. Wochenschr., 58, 323-330 (1921).

these mixtures, the values will be very close to each other: 74.6 dyns per cm. for oxalated, 74.8 for hirudinized plasma.

Let us compare in a similar way oxalated plasma and citrated plasma obtained from 4 guinea pigs. The surface tension of oxalated mixed plasma is 75.5, of citrated 75.4 dynes per cm.

The surface tensions of citrated and hirudinized plasma in dogs and in guinea pigs are consequently higher than the surface tension of water, and their values do not deviate at all from that of oxalated plasma.

We collected 9 cc. of blood from 4 patients between 21-25 years of age, in the morning, on an empty stomach, mixing it with 1 cc. of a physiological oxalated solution, then 9 cc. of blood in 1 cc. of citrated physiological solution, or hirudinized physiological solution, or even two portions of citrated blood and hirudinized blood.

As shown in the results in Table II, the values are almost identical for the surface tension of citrated plasma, hirudinized and oxalated plasma taken from the same dog or the same person.

TABLE II.

Kind of Animal	Surface Tension in Dynes per cc. at 18° C. for Plasma Diluted 1:10 per Volume		
	1 per Cent Sodium Oxalate and 0.65 per Cent NaCl	3.55 per Cent Sodium Citrate	0.85 per Cent Solution of Hirudinized NaCl
Dog	74.6	74.8	75.2
	75.0	74.9	...
	75.3	75.2	...
	75.6	75.9	76.1
	76.1	76.3	...
	76.4	76.5	76.7
Man	74.7	...	74.4
	74.9	75.2	...
	75.2	...	74.8
	75.7	75.9	76.2

The dynamic surface tension of plasma in a normal state does not seem to be modified noticeably by the addition of $\frac{1}{10}$ of its volume of one or another fluid anticoagulant.

These values remain constant for several hours, providing the blood has been taken from a fasting animal, properly collected, and immediately centrifuged at high speed for a long enough time, so as to obtain a very limpid plasma practically free from platelets and without any hemoglobin.

Kopaczewski[23] has recently criticized the use of citrated, hirudinized or oxalated plasma in experiments on modification of surface tension in different morbid states. He states as follows: "On reflection, it seems that plasma treated with these chemical substances ceases to be plasma. Because it does not coagulate; because its capillary properties are modified. And let us not forget, that the surface tension is precisely a capillary factor! What happens

[23] Kopaczewski, W., "Pharmacodynamie des colloïdes," Paris, II, 191 à 192 (1925).

to the plasma—a complex labile colloid—after addition of citrate, oxalate and hirudine? We do not know anything about it."

Based upon these considerations Kopaczewski believes that serum alone is a natural product. He is inclined to consider plasma (citrated, oxalated and hirudinized) as a somewhat artificial product. Therefore, for the measurement of surface tension, Kopaczewski decidedly prefers serum to plasma with addition of anticoagulants. Sachs and von Oettingen [24] are entirely opposed to this opinion, and due to the comparative investigations of M. La Barre on the surface tension of serum and plasma from the guinea-pig, dog and man (it is the same with the cat and rabbit) led me to make reply.[25] We agree with Sachs and von Oettingen that despite the addition of an anticoagulant the plasma, rather than the serum, represents the true blood medium existing during life.

Serum differs essentially from plasma by the absence of fibrinogen, that is, of that blood protein which is the most labile constituent and consequently most apt to undergo physico-chemical modifications. But coagulation does not consist of a simple transformation of fibrinogen from sol to gel. Fibrin, at the time of its formation, adsorbs H and Ca ions. The concentration of H and Ca ions in serum is smaller than in plasma, which yields the serum. Furthermore, it seems very probable that the transformation of fibrinogen into fibrin, or more correctly the coagulation, is preceded by a stage of precoagulation, during which the particles of fibrinogen are adsorbed by micells, either of serozyme or thrombin.[26]

The differences between the chemical constitution of plasma and of serum show why it is easier to comprehend the physico-chemical changes of the blood milieu in plasma than in serum.

The determination of the surface tension in serum seems besides to be subjected to more errors, than in properly collected plasma. There are many factors which tend to lower the surface tension of serum. In order to obtain exact results, speedy separation of serum from clot is advisable. It is also important to discard all cloudy or hemolyzed serum.[27] The surface tension of serum undergoes a gradual lowering, lasting sometimes for a long time, even when collected and preserved with extreme precautions, as demonstrated by Lecomte de Noüy [28] and Kopaczewski.[10]

According to Kopaczewski's criticism it would seem important to attempt to determine the surface tension of unchanged plasma without anticoagulants, and to compare it with the same plasma mixed with $\frac{1}{10}$ of its volume of a solution of sodium citrate or oxalate. This is unfortunately impossible under normal conditions and can be realized only under conditions which render blood uncoagulable. This is the case in acute anaphylactic shock, or in peptone shock in dogs, which are very suitable for this purpose, as will be seen later.

[24] Sachs, H. and von Oettingen, E., "Zur Biologie des Blutplasmas," *Münch. med. Wochenschr.*, 68, 351-353 (1921).
[25] Zunz, E. and La Barre, J., "Recherches sur la tension superficielle du plasma et du sérum humain à l'état normal et dans la syphilis," *Bull. Acad. roy. Méd. Belgique*, 5ᵉ série, IV, 34-107 (1924).
[26] Kugelmass, I N., "Contribution physico-chimique au mécanisme de la coagulation du sang," *Arch. int. de Physiol.*, 21, 139-190 (1923).
[27] Iscovesco, H., "L'influence de l'hémoglobine sur la tension superficielle," *Compt. rend. Soc. Biol.*, 70, 11-12 (1911), Kisch, B. and Remertz, O., "Ueber die Oberflächenspannung von Serum und Liquor cerebrospinalis beim Menschen und über die Technik Kapillarmetrischen Messungen," *Münch. med. Wochenschr.*, 41, 1097-1099 (1914); "Kapillarmetrischen Untersuchungen am Serum und Liquor cerebrospinalis des Menschen," *Int. Z. physikal. chem. Biol.*, 1, 354-388 (1914).
[28] Lecomte du Nouy, P., "Spontaneous decrease of the surface tension of serum," *Journ. of exper. med.*, 35, 575-597 (1922); "Chute spontanée de la tension superficielle du sérum et de ses solutions," *Compt. rend. Soc. Biol.*, 89, 1015-1017 (1923)

IV. LOWERING OF THE SURFACE TENSION OF PLASMA IN ANAPHYLACTIC SERUM SHOCK

Since 1913 I have demonstrated the lowering of the surface tension of serum collected 6 hours after reinjection of bovine serum into dogs.[29] But these animals do not show shock to a marked degree, and the influence of successive bleedings surely interfered with these relatively slight modifications. Continuing this research I collected blood during marked anaphylactic shock from dogs, which succumbed 3-4 hours after reinjection with bovine serum. A considerable lowering of the surface tension of both serum and oxalated plasma was noticed under these conditions. This lowering proceeded progressively and became more marked until the moment of death. The phenomenon was more pronounced in plasma than in serum.* Kopaczewski[30] observed in 1919 the lowering of the surface tension of serum in guinea pigs, which succumbed to shock caused by intravenous injection of homologous serum treated with colloidal gels.

It was important to find out at which point this symptom is a sign of anaphylactic or anaphylactoid shock. Together with M. La Barre,[31] I also had begun a new study of the physico-chemical modifications of blood during these two kinds of shock. We experimented with guinea-pigs, weighing between 250-350 grams, which show very rapid and violent reactions and are therefore particularly adapted for this research. But it is impossible to bleed the same animal repeatedly, because of the quantity of blood taken and the influence upon the animals of bleeding (even slight) during relatively small intervals of time. In order to avoid this error, it seemed to us necessary to compare the series of new guinea-pigs with animals which had been bled only once before.

We thus were able to show that carotid blood taken from a guinea-pig during anaphylactic shock several minutes after a provocative injection (reinjection of horse serum into guinea-pigs subjected to preliminary intraperitoneal treatment for 3 weeks), furnishes an oxalated plasma with a distinctly lowered surface tension.

The surface tension drop may amount to 10 dynes in violent shock caused by a quantity of serum exceeding the minimum lethal dose, and 8 dynes in the case of shock caused by the minimum lethal dose. When the injection of serum causes death, the average lowering of the surface tension of plasma is more marked in proportion to the injected dose of serum; it averages 5 dynes. Whenever the injection of serum produces a slight shock the average lowering of the surface tension of plasma is 3 dynes. Very small amounts of serum (0.001-0.05 cc.), which do not cause any symptoms of shock, do not modify the surface tension at all; its average value is, however, a little lower than the one which has been observed in fresh control guinea-pigs.

Table III illustrates clearly the relation between the degree of lowering of the surface tension of plasma and the intensity of anaphylactic shock.

[29] Zunz, E., "Recherches sur les modifications physico-chimiques du sang au cours de l'anaphylaxie," Z. Immunitätsf. exper. Therap., 18, 47-62 (1913).
* These experiments have been carried out by means of the stalagmometric method of Traube, which causes considerable errors, avoided in Kopaczewski's tonometric procedure. I repeated my experiments later, using the tonometer of Kopaczewski.
[30] Kopaczewski, W., "Le rôle des phénomènes physiques dans la production du choc anaphylactique," Compt. rend. Soc. Biol., 82, 590-593 (1919).
[31] Zunz, E. et La Barre J., "Sur les modifications physico-chimiques du sang lors du choc anaphylactique,"Compt. rend. Soc. Biol., 86, 286-288 (1922); "Recherches sur les modifications physico-chimiques du sang lors du choc anaphylactique sérique et du choc anaphylactoïde provoqué par l'injection intraveineuse de sérum traité par l'agar," Arch. int. Physiol., 21, 361-402 (1923).

TABLE III.

Dose of Horse Serum Injected into the Jugular Vein after the Provocative Injection	Surface Tension of Plasma in Dynes/cm. at 18° C.		
	Minimum Value	Maximum Value	Average Value
Exceeding the minimum positively lethal dose (0.18-0.4 cc.)	65.6	72.5	70.2
Minimum dose positively lethal (0.18-0.2 cc.)	69.2	72.4	70.4
Dose sometimes lethal, followed by symptoms:			
Grave (0.04-0.1 cc.)	67.8	72.5	70.5
Slight (0.1 cc.)	71.1	73.5	72.4
Non-lethal dose followed by slight symptoms (0.015-0.1 cc.)	71.5	73.3	72.0
Not followed by any symptoms (0.001-0.05 cc.)	74.0	75.4	74.9

The intensity of shock and the more or less marked degree of the surface tension drop are not absolutely proportional to the more or less venous appearance of the blood during anaphylactic shock in the guinea-pig. A marked lowering of the surface tension of the plasma of this animal can already be observed while the carotid blood still has its arterial appearance.

The lowering of the surface tension of plasma during anaphylactic shock from serum can be well seen in the dog, which does not show the least symptoms of asphyxia, and whose carotid blood still retains its arterial aspect. The surface tension of oxalated plasma from normal dogs weighing 10-15 kilograms, is not modified by taking successively every 20-30 minutes 10 cc. of blood, while, on the other hand, oxalated plasma from carotid blood taken during anaphylactic shock 20-90 minutes after an intravenous injection of horse serum into dogs, which received 3-6 weeks previous 10 cc. of the same horse serum intraperitoneally, has a surface tension sometimes as low as 60-69.3 dynes, i.e., is lowered by 5.5-6 dynes.

It can therefore easily be seen that asphyxia does not appreciably modify the surface tension of oxalated plasma.[32]

If, in fact, we tie up the trachea in guinea-pigs and after $1\frac{1}{2}$-3 minutes collect blood from the carotid, the animal becomes completely asphyxiated. The surface tension of its plasma varies between.74.4 and 74.9 dynes per cm.; that is, fairly normal values.

On saturating arterial blood from a normal animal (dog or guinea-pig) with carbon dioxide, the surface tension of its oxalated plasma will not be modified to any considerable extent. By passing oxygen through the asphyxiated carotid blood of a pretreated guinea-pig a few minutes after reinjection with horse serum, and by immediately centrifuging it, an oxalated plasma will be obtained which has a surface tension lowered in the same proportion as that obtained from other guinea-pigs pretreated in a similar way and reinjected simultaneously, but whose blood is centrifuged without previously passing oxygen through it.

The surface tension of oxalated plasma from the carotid blood of guinea-

[32] Zunz, E. et La Barre, J., "Observations complémentaires sur les modifications de la tension superficielle du plasma lors de l'anaphylaxie," *Compt. rend. Soc. Biol.*, 92, 223-224 (1925).

pigs and dogs remains normal during the period elapsing between pretreating injections and provocative injections. The average surface tension of guinea-pig plasma is equal to 75.6 dynes per cm. (the highest values being 74.5-75.9 dynes) three weeks after the intraperitoneal injection of 1-2 cc. of horse serum.

The surface tension of plasma is lowered in passive anaphylaxis of the guinea-pig in a similar way as in active anaphylaxis.[33] In both cases the surface tension is lower the more intense the shock.

Investigation of the surface tension of plasma helped me in determining whether proteoses and peptides have anaphylactogenous properties (sensitizing and provocative).[34]

The main conclusions which can be drawn from these experiments can be summarized as follows:

1. Six to eight weeks after an intraperitoneal injection of horse serum, an intravenous injection of heteroalbumose will produce intense anaphylactic shock with marked lowering (5-6 dynes) of the surface tension of plasma.

2. Six to eight weeks after an intraperitoneal injection with ox serum, intravenous injection with protoalbumose may produce a slight shock with a moderate lowering (2 dynes) of the surface tension.

3. Six to eight weeks after an intraperitoneal injection with heteroalbumose, intravenous injection of horse serum provokes intense anaphylactic shock with marked lowering (5 dynes) of the surface tension of plasma.

4. Six to eight weeks after intraperitoneal injection of a heteroalbumose, intravenous injection of this proteose, or of a protoalbumose, can lead to a slight shock with a lowering (of more than 3 dynes) of the surface tension of plasma.

5. Six to eight weeks after an intraperitoneal injection of proteoalbumose a slight shock and a slight lowering of the surface tension (2 dynes) of the plasma will be obtained by means of the same proteose, or by horse serum, and an intense shock with marked lowering (5 dynes) of the surface tension of the plasma will be produced by the protoalbumose.

6. Diglycylglycine and tetraglycylglycine do not sensitize the guinea-pig and do not produce anaphylactic shock.

I was thus able to confirm the sensitizing action of heteroalbumose and protoalbumose in the guinea-pig, to which fact I had already drawn attention in 1911.[35] I could, besides, corroborate the absence of sensitizing and provocative properties of diglycylglycine and tetraglycylglycine in the guinea-pig, as has already been stated by Abderhalden and Weil.[36]

Lumière and Couturier [37] believe that the cerebral nerve centers are indispensable for the manifestation of shock. This does not conflict with previous results, obtained by Schürer and Strassmann [38] and Pearce and Eisenberg.[39] Taking up the study of this problem with Mr. la Barre, I investigated, inci-

[33] Zunz, E. et La Barre, J., "Sur les modifications de la réaction et de la tension superficielle du plasma dans l'anaphylaxie passive," *Compt. rend. Soc. Biol.*, 90, 658-660.

[34] Zunz, E., "Nouvelles recherches sur l'anaphylaxie par les protéoses et les autres produits de désintégration des protéines," *Bull. Ac. roy. méd. Belgique*, 5ᵉ série, 3, 426-449 (1923).

[35] Zunz, E., "A propos de l'anaphylaxie," *Bull. Ac. roy. Méd. Belgique*, 4ᵉ série, 25, 425-461 (1911); "Recherches sur l'anaphylaxie par les protéoses," *Z. Immunitätsf. exper. Therap.*, 16, 580-619 (1913); Zunz, E. and György, P., "A propos de la toxicité des protéoses et de l'anaphylaxie par ces composés," *ibid.*, 23, 296-305 (1914).

[36] Abderhalden, E. and Weil, A., "Versuche über das Wesen der Anaphylaxie," *Z. physiol. Chem.*, 109, 289-297 (1920).

[37] Lumière, A. and Couturier, H., "Sur le rôle du système nerveux dans les chocs anaphylactiques," *Arch. int. Pharmacodyn. et Thérap.*, 31, 265-277 (1916).

[38] Schürer, J. and Strassmann, R., "Zur Physiologie des anaphylaktischen Shockes," *Z. Immunitätsf. exper. Therap.*, 12, 143-152 (1912).

[39] Pearce, R. M. and Eisenberg, A. B., "The physiology of anaphylactic shock in the dog," *J. infect. Dis.*, 7, 565-576 (1910).

dently, the problem, whether the lowering of the surface tension of plasma, so characteristic of anaphylactic shock, occurs also after reinjection of decerebrated guinea-pigs with horse serum 3 to 4 weeks after the preliminary injection. In order to solve this problem I measured, together with La Barre,[40] the surface tension of oxalated plasma obtained from carotid blood taken ½-2 minutes after the provocative injection. We made this determination partly with pretreated guinea-pigs after the removal of their brains, partly with fresh guinea-pigs, operated upon in a similar way. Finally intravenous injections with horse serum were carried out 1-2 hours after the operation, in order to avoid the influence of the anesthetics. The plasma of the control decerebrated guinea-pigs had under these conditions a lower dynamic surface tension than normal, averaging 73.3 dynes per cm. at 18° C. (extreme variations 72.9-73.7) instead of 75.7 dynes (extremes 74.3-76.6) under normal conditions. The carotid blood of decerebrated pretreated guinea-pigs taken shortly after the reinjection of serum has a marked venous appearance. The average surface tension of oxalated plasma in intense shock is 70.1 dynes (death in 8-10 minutes) and 67.6 dynes after violent shock (death in 2-3 minutes).

The surface tension is therefore considerably lowered after the reinjection of serum into decerebrated guinea-pigs. This lowering averages 4.5 dynes, when compared with the values obtained in control decerebrated animals. The injection of a positively lethal dose of serum into guinea-pigs lowers the average surface tension of their plasma to 70.2 dynes, and sometimes to 65.7 dynes.

The animals from which the brain and the medulla have been removed simultaneously, show an analogous lowering of the surface tension during the anaphylactic crisis.

We are therefore induced to conclude that carotid blood taken during an anaphylactic crisis from decerebrated guinea-pigs, shows a drop in surface tension, indicating the existence of important modifications in the physico-chemical and colloidal equilibrium of blood plasma, observed as following the state of shock.

The plasma of decerebrated cats has a dynamic surface tension, which is somewhat below normal, being 72.5 dynes per cm. at 18° C.

These values are not modified by taking first 10-15 cc. of blood 10-90 minutes before. When the blood is taken too soon after decerebration, the surface tension may become as low as 70.6 dynes per cm., due to the influence of ether, which has not yet been completely eliminated. It is also necessary to prolong artificial respiration for at least 15-20 minutes after the operation, before taking blood from pretreated decerebrated cats.

The carotid blood collected during the maximum drop of the arterial pressure in decerebrated pretreated cats, gives a plasma with a surface tension as low as 69 dynes per cm. The surface tension during fatal anaphylactic shock may be lowered in the cat by 4.5-5 dynes when compared with values obtained in decerebrated control animals, or after a provocative injection in prepared cats.

We are therefore justified in comparing the values obtained from the cat with those obtained with guinea-pigs under analogous conditions.

With regard to Kopaczewski's [23] criticism of the use of citrated oxalated

[40] Zunz, E. and La Barre,, J., "Modifications de la coagulabilité et de la tension superficielle du plasma lors de l'anaphylaxie sérique chez des cobayes décérébrés," *Compt. rend. Soc. Biol.*, **95**, 858-860 (1926); "Sur la persistance des phénomènes du choc anaphylactique chez le cobaye et chez le chat décérébrés," *Arch. int. Pharmacodyn et Thérap.*, **32**, 255-268 (1926).

plasma for the determination of surface tension, as described above, it seems necessary to inquire, whether there is really produced a lowering of the surface tension of plasma after the provocative injection. In order to solve this problem, it would be necessary to determine the surface tension of plasma without the addition of sodium oxalate, or citrate, and to compare it with that of citrated, or oxalated plasma, collected under the same conditions.

Acute anaphylactic shock from serum in the dog is perfectly suitable for this experiment. Suppose we inject into the jugular vein of a dog weighing 10-15 kilograms, 1 cc. of horse serum per kilogram of body weight after the dog had received 3-6 weeks previous the same quantity of serum subcutaneously. The carotid blood, which in most of the cases will still be perfectly red when collected 10-90 minutes after the reinjection with foreign serum, gives on immedate and prolonged centrifuging a clear, spontaneously incoagulable serum, the surface tension of which may be easily determined by Kopaczewski's tonometric method. Nine cc. of blood, obtained from each bleeding during anaphylactic serum shock, are then added to 1 cc. of physiological oxalated or citrated solution, or to a physiological salt solution. The different blood mixtures (citrated, oxalated, and physiological salt solutions) are centrifuged simultaneously. Before reinjection with horse serum 10 cc. of citrated blood and then 10 cc. of oxalated blood are collected and the order of the different blood specimens carefully varied. This does not modify the surface tension of citrated or oxalated plasma obtained from carotid blood specimens collected 10-120 minutes later.

Here are the results of 5 experiments [41] made this way:

TABLE IV.

Dog Used in Experiment	Moment of Experiment	Surface Tension of Plasma in Dynes per cm. at 18° C.			
		Found for the Plasma Itself	Diluted by $\frac{1}{10}$ of Its Volume:		
			0.9 per Cent NaCl	1 per Cent Sodium Oxalate and 0.65 per Cent of NaCl	3.55 per Cent Sodium Citrate
I	Before reinjection ..	...	...	76.1	76.3
	45 min. after	70.7	...	71.8	71.4
II	Before reinjection ..	...	...	75.3	75.2
	10 min. after	73.4	...	74.4	73.8
	55 min. after	73.0	...	73.7	73.5
III	Before reinjection ..	...	...	...	75.2
	40 min. after	70.1	...	70.7	70.4
	90 min. after	72.7.	...	73.1	73.2
IV	Before reinjection ..	...	...	...	75.1
	20 min. after	69.7	...	71.0	70.8
V	Before reinjection ..	...	...	75.0	74.9
	15 min. after	73.6	...	73.8	73.2
	50 min. after	71.9	72.5	72.3	72.7
	90 min. after	68.2	69.9	70.1	69.7

[41] Zunz, E., "De la tension superficielle du plasma de choc spontanément incoagulable," *Compt. rend. Soc. Biol.*, **93**, 463-465 (1925). Zunz, E. and La Barre, J., "Contribution à l'étude des modifications de

The data collected in Table IV show a gradual lowering of the surface tension of undiluted plasma during the course of acute anaphylactic serum shock in the dog.

This phenomenon is more marked for the plasma itself, than for citrated or exalated plasma. When, in fact, the surface tension of citrated or oxalated plasma is lower than that of water (73 dynes per cm. at 18° C.), the surface tension of pure plasma is a little lower than that of the same plasma diluted with psysiological solution, or citrate or oxalate. This is certainly partly due to the surface tension of the diluting fluid itself: 72.6-72.8 dynes for physiological salt solution, 73.2-73.5 for a solution of citrate, and 74.6-74.8 for a physiological solution of oxalate.

The surface tension of spontaneously incoagulable plasma in shock is thus obviously lowered. During this manifestation the addition of sodium citrate or oxalate does not seem at all to modify the capillary properties of plasma.

Briefly the lowering of the surface tension of citrated or oxalated plasma during acute anaphylactic shock in dogs may be considered as a definite fact, because it is also observed in unchanged plasma. Everything seems, besides, to indicate that the surface tension of unchanged plasma in the normal dog always exceeds that of water and approaches to a greater or less degree the values obtained with citrated or oxalated plasma.

V. The Lowering of the Surface Tension of Plasma in Anaphylactoid Shock

The lowering of the surface tension of citrated or oxalated plasma is encountered not only in anaphylactic serum shock, but also in different conditions of anaphylactoid shock. Let us consider some of these cases.

A. Injection of Fresh Guinea-pigs with Homologous Serum Treated with Agar

The intravenous injection of guinea-pigs' serum treated with agar, according to Bordet's [42] method, causes in a guinea-pig symptoms analogous to those observed in anaphylactic shock. This is not the case when the serum is kept for 20-30 minutes at a temperature of 58° before the addition of agar, which is removed after 2 hours of contact at 38°.

Table V shows the lowering of the surface tension of plasma obtained from carotid blood of fresh guinea-pigs which were injected a few minutes before with the minimum postively lethal dose, or with an excessive dose of serum from guinea-pigs pretreated with agar, according to Bordet's method.[43] This phenomenon is the more pronounced, the more toxic the serum treated with agar appears. The lowering is sometimes less intense than in acute anaphylactic shock in guinea-pigs pretreated with horse serum. It also takes place, but in a lesser degree, when using agar-treated serum in doses not necessarily fatal, or in doses producing more or less pronounced symptoms of shock without ever terminating in death.

But even when injecting the same amount of serum, relatively considerable differences can sometimes be observed in different guinea-pigs. The lowering

la coagulation du sang au cours du choc anaphylactique chez le chien," *Arch. int. Physiol.*, **25**, 221-264 (1925).

[42] Bordet, J., "Le mécanisme de l'anaphylaxie," *Compt. rend. Soc. Biol.*, **74**, 225-227 (1913); "Gélose et anaphylaxie," *Bull. Soc. Roy. des Sc. Méd. et Nat. de Bruxelles*, **71**, 25-37 (1913).

[43] Zunz, E. and La Barre, J., "Sur les modifications physico-chimiques du sang lors de l'injection de sérum traité par l'agar," *Compt. rend. Soc. Biol.*, **87**, 805-807 (1922); *Arch. int. de Physiol.*, *loc. cit.* 31.

The surface tension of citrated blood during peptone shock becomes lowered in a variable degree: very weak (1.7 dynes) in experiment I, very marked on the contrary (9.4-9.2 dynes) in experiments II and III, less intense, but still noticeable (5.5 and 5.1 dynes) in experiments IV and V.

When the surface tension of citrated plasma is lower than that of water (73 dynes per cm. at 18°) the surface tension of the unchanged plasma is somewhat lower than the same plasma diluted with physiological solution, or with oxalate, or citrate. In experiment I, in which the surface tension of citrated plasma exceeds that of water by one dyne, the surface tension of undiluted plasma is somewhat higher than that of citrated plasma, while the surface tension of oxalated plasma is higher than that of undiluted plasma, and the surface tension of plasma diluted with physiological solution is inferior to that of plasma itself. These slight differences depend particularly upon the surface tension of the diluting fluids: 72.6-72.8 dynes per cm. at 18° for physiological solution (72.7 in experiment I), 73.2-73.5 for citrated solution (73.4 in experiment I), 74.6-74.9 for the oxalated physiological solution (74.9 in experiment I).

The addition of citrate or oxalate does not seem, in fact, to change the surface tension of spontaneously incoagulable plasma, taken from the dog during peptone shock. The surface tension of untreated plasma, at least for this species of animals, certainly under normal conditions exceeds that of water. These facts are in perfect agreement with the observations made during the acute anaphylactic serum-shock of dogs.

The results of experiment I are of a particular interest. They prove, in fact, that a plasma might not coagulate spontaneously and still show a slight lowering of the surface tension. We might mention that this animal survived, like the four other dogs which received Witte's peptone.

C. Intravenous Injection of Histamine

After injecting a guinea-pig intravenously with histamine, the surface tension of its plasma decreases by 3-4 dynes at a maximum.[47] This phenomenon is less intense than that observed during acute anaphylactic shock in the guinea-pig, when the lowering of the surface tension may attain 9 dynes.

A lowering of the surface tension of plasma is observed in the dog only after intravenous injection of a very large dose of histamine, say 3 mgr. per kilogr. of body weight. It then becomes 5 dynes and exceeds that observed in the guinea-pig during violent histamine shock, produced by 2 mgrs. of histamine per kgr. of body weight. But while in the guinea-pig 0.2 mgr. of this substance are already sufficient to lower the surface tension, a 10 times larger dose, for example 2 mgr., does not change the surface tension in the dog.[48]

D. Intravenous Injection of Électrargol

In normal man on fasting, or several hours after a light meal, the oxalated plasma from blood taken from the bend of the elbow has a surface tension of 74.1-76.7 dynes per cm. When the same person is bled twice in succession after an interval of 30-60 minutes, no appreciable change of the surface ten-

[47] La Barre, J., "Sur les modifications du pH du plasma lors du choc histamitique et ses rapports avec l'abaissement de la tension superficielle," *Compt. rend. Soc. Biol.*, **95**, 237-238 (1926).
[48] Zunz, E. and La Barre, J., "Tension superficielle et coagulation du plasma lors du choc histamitique du chien," *Compt. rend. Soc. Biol.*, **96**, 722-724 (1921).

sion is observed. It is also not influenced even by a relatively high rise of temperature (39-40°), when no other factors intervene.

A rapid intravenous injection of 5-10 cc. of electrargol causes in man a condition of shock, characterized by violent chills, dyspnea, a rapid lowering of arterial pressure, an increase of pulse frequency, and a rise of temperature, reaching its maximum within 30-60 minutes. The temperature then drops below normal and often remains low for several days.

I observed, together with M. La Barre,[49] some patients subjected to this treatment, and took their blood before and after treatment. We observed a lowering of the surface tension of the plasma during the course of shock thus provoked. This lowering may attain 4-5 dynes per cm. when the blood is taken during the moment of the maximum temperature rise, that is ½ hour-1½ hours after injection. One half of an hour after the maximum temperature rise, the surface tension of the plasma sometimes still shows a lowering of 2.5 dynes per cm.; 48 hours after the intravenous injection of collargol there is usually again a normal surface tension. The lowering of the surface tension seems to be dependent upon the intensity of shock.

The lowering of the surface tension after the intravenous injection of electrargol thus demonstrates that this therapeutic agent, besides producing clinical symptoms, also causes profound changes of the colloidal equilibrium of the blood.

When injecting a guinea-pig with 0.5-1 cc. of electrargol (per 250 grams of body weight) into the jugular vein, there can be observed after 10-30 minutes a considerable rise of the rectal temperature, also a marked polypnea, followed by excitement and slight twitching. The dyspnea and the muscular twitching sometimes appear even after 5-7 minutes. These symptoms persist for a variable length of time, and then everything returns to normal. The guinea-pig recovers perfectly after these doses.

Suppose we take some carotid blood at the moment of appearance of these symptoms. The surface tension of the plasma generally is lowered by 3-4 dynes per centimeter. In exceptional cases it may even drop to 68.7 dynes. Generally the drop in the surface tension of plasma becomes more marked in proportion to the increase of the dose of the injected electrargol, and the observed symptoms more marked.

The intravenous injection of 0.3 cc. of electrargol (per 250 gr. of body weight) do not cause any symptoms in the guinea-pig. The carotid blood taken 10-30 minutes after this injection gives a citrated or oxalated plasma with a normal surface tension.

E. INTRAVENOUS INJECTION OF NOVOARSENOBENZOL

There are sometimes observed in the man, after the intravenous injection of novoarsenobenzol, phenomena which resemble those produced by the introduction of electrargol into the blood.

Thanks to Mr. Govaerts's kindness, I was able to examine citrated plasma collected under such circumstances. The surface tension was 65.9 dynes per centimeter at 18° C. Citrated blood was taken twice in succession in the morning before breakfast. The surface tension of plasma from this blood was 69.5-69.6 dynes. It should be understood that these are not normal figures, because they have been obtained from a syphilitic with a strongly

49 Zunz, E. and La Barre, J., "Abaissement de la tension superficielle du plasma après l'injection intra-veineuse d'électrargol," Compt. rend. Soc. Biol., 90, 118-120 (1924).

positive Bordet-Wassermann. I have, however, been able to find a lowering of more than 3.5 dynes during shock from arsenobenzol.

It can be stated definitely, that a lowering of the surface tension of plasma can be observed during different states of shock, as studied in guinea-pigs, dogs, and in man. This is certainly a very important proof of the changes of the colloidal equilibrium of plasma, occurring during the course of anaphylactic or anaphylactoid shock, but the lowering of the surface tension can no more, than the other changes observed in the blood, be considered as the cause of the conditions of shock.[50] The lowering of the surface tension of plasma under these circumstances, shows simply the profound changes of the humoral medium resulting fom anaphylactic, or anaphylactoid shock, as already stated by Nolf [51] and by other authors.

VI. THE ANTISHOCK ACTION OF ATROPINE, CHOLINE, HIRUDIN AND THE SURFACE TENSION OF PLASMA DURING THE STATE OF SHOCK

If the lowering of the surface tension is an important proof of the colloidal disequilibrium during shock, this phenomenon should become less pronounced or should disappear, whenever by preliminary injections of suitable substances the development of the resulting effects of the provocative injections could be either prevented or diminished. The experiments which we will now describe show that this is actually the case.

A. ANTISHOCK ACTION AND SURFACE TENSION OF PLASMA IN SERUM ANAPHYLAXIS

The intraperitoneal injection of a milligram of atropin sulfate (per 100 grams of body weight) prevents in about 80 per cent of the cases the anaphylactic shock produced by introduction 20-35 minutes later of the minimum positively lethal dose of horse serum into the jugular vein of a pretreated guinea-pig.[52] In about 20 per cent of the cases, a more or less severe shock is observed; the guinea-pig survives or slowly dies. If we inject several fatal doses of serum, more or less marked shock is observed, even after the intraperitoneal injection of atropin. If we inject 0.28-0.5 milligram of atropin sulfate, the positively lethal dose of serum will show its effects exactly like in control animals, which did not receive atropin. By intraperitoneal injection of 1-4 centigrams, or by intravenous injection of 1-2 centigrams of choline chlorhydrate, it is possible to a certain extent to protect a guinea-pig from shock produced by reinjection with horse serum.

This protection does not follow immediately after the injection of choline; it does not last for long and it occurs only with the minimum positively lethal dose of serum, or for a dose slightly larger. The intraperitoneal injection of 5 milligrams of choline chlorhydrate or smaller quantities of this substance, do not exert any protective action.

The best protection is obtained· after an intraperitoneal injection of 2 centigrams of choline chlorhydrate carried out 15-25 minutes before reinjection with horse serum. Under these conditions some guinea-pigs will not show any symptoms after the introduction of serum into the jugular vein. Others will show either slight or grave symptoms. Some will succumb after

⁵⁰ Zunz, E., "Considérations physico-chimiques sur l'anaphylaxie," *Bull. Soc. Roy. des Sc. Méd. et Nat. de Bruxelles,* 105-106 (1922).
⁵¹ Nolf, P., "La composition protéique du milieu humoral. III. De l'anaphylaxie," *Arch. int. de Physiol.,* 10, 37-77 (1910).
⁵² Zunz, E. and La Barre, J., "A propos de l'action protectrice de l'atropine dans le choc anaphylactique du cobaye," *Compt. rend. Soc. Biol.,* 91, 132-134 (1924).

several hours. Others, finally, will die as soon and with the same symptoms as the control animals, which did not receive choline.[53]

I showed, together with Mme. Geertruyden-Bernard,[54] that the intravenous injection of 2.5-5 milligrams of hirudin protects, in a rather variable way for the individual guinea-pigs, against the anaphylactic symptoms caused by reinjection of horse serum, provided, that between the injection of hirudin and that of serum, there elapses a period of from 2½ hours-4 to 6 hours. Under these conditions the minimum postively lethal dose of serum, or a slightly larger dose, usually produces very mild symptoms and death will follow only after several hours. Sometimes the animal will survive. A dose slightly smaller than the positively lethal dose will usually provoke only slight symptoms and the animal will survive. A preliminary injection of hirudin sometimes diminishes the effects of a dose of serum which is slightly in excess of the minimum positively lethal dose.

When only 5-15 minutes elapse between the injection of hirudin and that of serum, the anaphylactic shock is usually just as violent as in pretreated guinea-pigs which did not receive hirudin. It sometimes happens that, within the lapse of 5-20 minutes, hirudin may somewhat mitigate the effects produced by the reinjected serum, and death may follow only within ½ hour up to 10 hours. In exceptional cases the guinea-pigs survive after an injection corresponding to 5 times the minimum positively lethal dose for pretreated control animals which have not been subjected to a preliminary treatment with hirudin. This substance exerts its protective action in doses as small as 1.5-2 milligrams.

The injection of 2-4 milligrams of hirudin in untreated guinea-pigs causes a lowering of the surface tension of plasma amounting to about 2 dynes; it has a tendency to approach that of water still exceeding it with an average of about 0.5 dyne. This slight lowering of the surface tension is maintained only during the first 15 minutes following the injection; the surface tension of plasma then becomes normal.

The average value of the surface tension of oxalated plasma is equal to 75.7 dynes for fresh guinea-pigs, 75.4 dynes for guinea-pigs which had received 5-25 minutes previously choline chlorhydrate intraperitoneally, 75.5 dynes for guinea-pigs which had received 20-35 minutes before atropin sulfate, 75.5 dynes for guinea-pigs which have been injected with hirudin into the jugular 1½-3 hours before.

Table VII shows the changes of the surface tension of plasma, following the injection of a positively lethal dose of serum into pretreated guinea-pigs which had received atropin, cholin, or hirudin previous to the reinjection. The surface tension of oxalated plasma, obtained from carotid blood during marked shock provoked by a minimum postively lethal dose of horse serum, amounts, according to Table VII, to 70.4 dynes for pretreated guinea-pigs. It is equal to 69.0 dynes in guinea-pigs not protected by hirudin; to 70.1 in those not protected by choline, and to 70.3 in those which were not protected by atropin. Even after preliminary injection with atropin, choline, or hirudin, the diminution of the surface tension reaches an average of 5 dynes during serum shock and sometimes exceeds 6-8 dynes.

[53] Zunz, E. and La Barre, J., "Action anti-choc de la choline chez le cobaye dans l'anaphylaxie sérique," *Compt. rend. Soc. Biol.*, **88**, 654-656 (1923).
[54] Zunz, E. and Mme. Van Geertruyden-Bernard, "Recherches sur l'action de l'hirudine sur les accidents anaphylactiques et sur les effets de l'injection intraveineuse de sérum traité par l'agar," *Arch. int. de Physiol.*, **20**, 79-102 (1922).

TABLE VII.

Substance Injected Before Reinjection with Serum	Effect of the Preliminary Injection	Surface Tension of Plasma in Dynes/cm. at 18° C.		
		Minimum Value	Maximum Value	Medium Value
None	......	69.2	72.4	70.4
Atropine sulfate ..	No protection	70.1	70.5	70.3
	Incomplete protection	72.1	73.2	72.6
	Complete protection	74.6	75.5	75.1
Choline chlorhydrate	No protection	69.2	71.1	70.1
	Incomplete protection	72.6	73.3	72.9
	Complete protection	75.9	76.2	76.0
Hirudin	No protection	66.5	70.9	69.0
	Incomplete protection	71.2	73.9	72.6
	Complete protection	72.1	74.7	73.4

When the shock is considerably weakened by means of choline, the surface tension of plasma averages 72.9 dynes. It is slightly higher than the average surface tension of plasma (72.6) in guinea-pigs not completely protected by atropin or hirudin. A considerable weakening of the shock coincides thus, in the three cases, with the minimum lowering of the surface tension of plasma.

When the protection by means of choline is complete, that is, when no shock symptoms are noticeable, the surface tension of plasma is never lowered, when compared with the normal, and averages 76.0 dynes. Under these same conditions the surface tension of plasma from guinea-pigs treated by atropin varies within normal limits, still averaging somewhat lower (75.1 dynes) than in unused control guinea-pigs. In hirudinized guinea-pigs without shock, the surface tension of plasma is sometimes higher, sometimes lower than that of water, averaging slightly above, but remaining lower than normal (average lowering of 2 dynes). Should not this fact be considered as perhaps due to the slight lowering of surface tension produced by the hirudin in unused guinea-pigs shortly after its intravenous injection, as described above?

When injecting several lethal doses of serum into pretreated guinea-pigs, there appear more or less marked symptoms of shock, even after a preliminary injection of atropin, choline, or hirudin: the surface tension of plasma becomes more or less lowered, just as in control animals.

B. ANTI-SHOCK ACTION AND SURFACE TENSION OF PLASMA, DUE TO ANAPHYLACTOID SHOCK •

The chlorhydrate of choline has a protective action for the guinea-pig. Against the toxic effects of an intravenous injection of homologous serum treated with agar. Like in anaphylactic shock, due to reinjection with horse serum, this protection is neither immediate nor of a long duration, nor of a considerable intensity. A protective action is observed, particularly marked 20-30 minutes after the introduction of 2 centigrs. of chlorhydrate of choline into the peritoneal cavity. The protecting effect appears only rarely after 20 minutes; it begins to weaken after 10 minutes and soon disappears com-

pletely. Smaller doses (½-1 centigram) of chlorhydrate of choline are less effective. The toxic action of choline itself is felt from larger doses (3 centigrams or more).

When the intraperitoneal injection of 2 centigrams of choline chlorhydrate precedes the intravenous injection of homologous agar treated serum by 20-30 minutes, in about ⅔ of the cases a protection against the positively lethal doses of serum is obtained. This protection is sometimes complete, that is, no shock symptoms are observed, sometimes incomplete, that is, it produces mild shock with survival of the guinea-pig or death several hours afterwards. The protective action of choline chlorhydrate is generally effective for the minimum dose, sometimes lethal. It is exceptional, on the other hand, for a dose slightly larger than the minimum positively lethal dose.

The intravenous injection of 2.5-5 milligrams of hirudin in the guinea-pig always diminishes. The toxic effect of agar-treated serum, provided that at least 1½ hours have elapsed between the injection of hirudin and that of the serum. This diminution is sometimes quite marked.[54] It is still very marked when only 2 milligrams of hirudin per 250 grams of guinea-pig are injected, and when 2½-3 hours elapse before the introduction of the gelose-treated serum into the jugular.

Table VIII shows the values for the surface tension of guinea-pig plasma treated or with choline, or with hirudin previous to the intravenous injection of the minimum positively lethal dose of homologous agar treated serum, or of a slightly larger dose. Because of the different degree of toxicity of different specimens of agar-treated serum, the table shows also the values obtained in each series of experiments in control guinea-pigs during anaphylactic shock.

TABLE VIII.

Substance Injected Previous to the Agar Treated Serum	Effect of the Preliminary Injection	Surface Tension of Plasma in Dynes at 18° C.		
		Minimum Value	Maximum Value	Medium Value
First series of experiments .. {None Hirudin	No protection Complete protection	67.0 73.6	71.3 74.1	70.6 73.9
Second series of experiments .. {None Chlorhydrate of choline	No protection No protection Incomplete protection Complete protection	68.7 66.8 68.1 69.8	69.8 68.2 69.3 72.2	69.1 67.5 69.0 71.0

Hirudin afforded an absolute protection in the experiments, the results of which are given in Table VIII. A lowering of the surface tension of the plasma by about 2 dynes is, however, observed, similar to the observations made after the injection of serum first heated to 58° and only then treated by agar.

An intraperitoneal injection of 2 centigrams of cholin chlorhydrate does not prevent a lowering of the surface tension of plasma, when no protection against the toxic effects of an intravenous injection of homologous agar treated serum has been established.

Even when the protection seems complete, that is, when there are no shock symptoms, the surface tension of plasma remains less than that of

water, but is higher in guinea-pigs not protected by choline and in control animals not treated with this substance. It should, however, be pointed out that the lowering of the surface tension of plasma has been more marked in this series of experiments than in the others carried out with agar-treated homologous serum. Be this as it may, the absence of all shock symptoms coincides with a less marked lowering of the surface tension, than in control guinea-pigs treated with gelose serum.

The difference between the average surface tensions of plasma in control guinea-pigs and hirudinized guinea-pigs is more pronounced than between control animals and guinea-pigs injected with choline chlorhydrate.

Everything seems, besides, to indicate that hirudin protects unused guinea-pigs better than choline chlorhydrate, against anaphylactoid shock caused by intravenous injection of homologous serum treated with agar.

Because of the characteristic effects of the intravenous injection of a relatively large quantity of fluid (2-3 cc.) the influence of a preliminary protective injection upon the surface tension of plasma is less noticeable after the injection of agar-treated homologous serum in untreated guinea-pigs than after the reinjection of horse serum into pretreated guinea-pigs. The two series of experiments seem to show that the more or less marked degree of protection exerted by atropin, choline, or hirudin against shock, coincides with values of the surface tension of plasma, which approach more or less the normal values.

VII. Relations between the Reaction and the Surface Tension of Plasma

On injecting 0.015-0.4 cc. of horse serum into the jugular of guinea-pigs, which received 3-4 weeks before a preparatory injection of this serum intraperitoneally, the carotid blood taken during full shock, several minutes after the provocative injection, shows, besides the lowering of the surface tension of the plasma: (1) an increase of the viscosity of the whole blood, without an appreciable change of the viscosity of the plasma; (2) a slight increase of the refractometric index of the plasma; (3) a slight lowering of the freezing point of the plasma; (4) an increase of the number of the red cells and a corresponding decrease of the quantity of plasma.

All these different phenomena appear not only after the provocative injection of the minimum positively lethal dose, or of a larger dose, but also after the introduction of a smaller quantity of serum than the minimum positively lethal dose which is not followed by any symptoms into the jugular.

I investigated, together with Mr. La Barre,[55] whether the changes in the reaction of plasma obtained from carotid blood, taken during anaphylactic shock, are parallel to the lowering of the surface tension observed under these conditions. We therefore determined the pH colorimetrically, using an undiluted plasma, or plasma diluted with physiological solution, taking all the precautions described by Cullen.[56]

Our experiments were carried out, using as indicators phenolsulphophthalein (phenol red and dibromothymolsulphophthalein (bromothymol blue). Table IX shows the results obtained with guinea-pigs.

[55] Zunz, E. and La Barre, J., "Sur les modifications du pH du plasma lors du choc anaphylactique et ses rapports avec l'abaissement de la tension superficielle," *Compt. rend. Soc. Biol.*, 88, 990-991 (1923).
[56] Cullen, G. E., "Studies of acidosis, XIX. The colorimetric determination of the hydrogen ion concentration of blood plasma," *J. biol. Chem.*, 52, 501-515 (1922).

TABLE IX.

Quantity of Horse Serum Injected into the Jugular in cc.	Symptoms	pH	Surface Tension in Dynes/cm. at 18° C.
0.0	None	7.31–7.36	75.4–75.7
0.01	None	7.34–7.35	75.1–75.5
0.05	Light	7.26–7.32	71.8–72.7
0.05	Grave	6.90–7.16	71.0–71.4
0.1	Light	7.22–7.30	71.3–72.5
0.1	Grave	7.04–7.20	69.4–72
0.2	Grave	7.06–7.18	69.6–71.8
0.2	Violent	7.02–7.12	69.3–71.4
0.25	Very violent	6.90–7.04	68.7–70.4

As seen from Table IX, there is a lowering of the pH of plasma during the course of anaphylactic shock. The alkaline reaction of plasma decreases in proportion to the extent of the shock symptoms; it might even become slightly acid. The pH of plasma decreases, as a rule, the more the surface tension drops.

There is no absolute parallelism between the intensity of the lowering of the pH of the plasma and the amount of serum introduced into the circulation after the provocative injection, or the more or less venous aspect of the carotid blood taken during shock.

The quantity of horse serum, which does not cause shock symptoms, after reinjection, does not modify the reaction and the surface tension of the plasma.

The reaction of plasma is subject to analogous modifications during passive anaphylaxis. We succeeded in producing such a condition in the guinea-pigs A, which received 3 intraperitoneal injections of horse serum, that is, 0.5 cc. the first day; 0.5 to 1 cc. the second day; 1 cc. the third day. Three weeks later the state of anaphylaxis was present, as verified in several of them: An intravenous injection of 0.2 cc. of horse serum provoked, in fact, rapidly fatal shock. We then bled the remaining guinea-pigs A. We centrifuged their blood immediately at high speed in order to obtain their serum, and we injected 3-4 cc. of the latter into untreated guinea-pigs B. From 6 hours to 3 days after this, we injected 0.3-0.6 cc. of horse serum into the jugular of the guinea-pigs B, thus prepared. Table X shows the reaction and the surface tension of the plasma in these animals.

TABLE X.

Quantity of Horse Serum Injected into the Jugular in c.c.	Symptoms	Aspect of Blood	pH	Surface Tension in Dynes/cm. at 18° C.
. . . . . .	None	Red	7.38–7.43	74.8–75.6
0.3–0.4	None	Red	7.32–7.46	74.7–75.7
0.4–0.5	Light	Red	7.36–7.40	73.6–74.8
0.4–0.6	Grave	Venous	7.28–7.33	72.2–73.2
0.5–0.6	Violent	Venous	7.16–7.18	71.9–72.1
0.5–0.6	Very violent	Very venous	6.88–7.12	68.7–71.1

The reaction of the plasma of guinea-pigs injected with choline chlohydrate intraperitoneally or with hirudin intravenously before the reinjection with

horse serum, remains normal, when the protection is complete. The pH of the plasma is, however, lower to a greater or less extent, and is about parallel with the diminution of the alkalinity. Contrary to what happens in anaphylactic serum shock, there is no absolute parallelism between the value of the pH of the plasma and the degree of the lowering of its surface tension. A plasma having a very much lowered surface tension may, in fact, have a normal pH.

The intensity of the lowering of the pH of the plasma does not, besides, depend upon the more or less venous aspect of the carotid blood collected during shock.[57]   This can easily be seen from Table XI.

TABLE XI.

Quantity of Serum Treated with Agar Injected into Jugular in cc.	Symptoms	pH	Surface Tension in Dynes/cm. at 18° C.
0.0	None	7.32–7.40	75.4–75.9
0.05	None	7.31	75.5
0.1	None	7.42	76.0
0.25	None	7.32	73.9
0.5	None	7.32–7.35	73.7–75.8
1.0	Light	7.36	73.9
1.2	Grave	7.15	72.9
2.0	Grave	7.32	73.5
2.5	Grave	7.38	71.9
3.0	Violent	7.08–7.40	70.8–71.4
3.5	Violent	7.22–7.36	69.8–70.6
4.0	Very violent	7.06	70.1

After an intravenous injection of Witte's peptone or histamine, there can also be observed in the guinea pig a tendency of the reaction of the plasma to become more neutral, the more violent the shock becomes.

The same takes place with citrated or oxalated plasma obtained from carotid blood of a guinea pig collected 10–30 minutes after the intravenous injection of 0.5–1 cc. of electrarzol.   The pH of the plasma may become as low as 7.2.[58]   The reaction is, in general, more alkaline the larger the injected dose of electrargol and the higher the rectal temperature of the guinea-pig.

VIII. Relation Between the Velocity of Sedimentation of the Red Cells and the Surface Tension of Plasma

Löhr [59] found an absence of the sedimentation of the red cells in man during anaphylactic shock followed by a marked acceleration after the disappearance of the shock symptoms, followed by a return to the normal on about the 6th day.

Witkower [60] reports a great decrease of the velocity of the sedimentation

[57] Zunz, E. and La Barre, J., "Sur les modifications du pH du plasma après l'injection de sérum traité par l'agar et ses rapports avec l'abaissement de la tension superficielle," *Compt. rend. Soc. Biol.*, 89, 676-678 (1923).

[58] La Barre, J., "Sur les modifications du pH du plasma après l'injection, intraveineuse d'électrargol chez le cobaye," *Comp. rend. Soc. Biol.*, 90, 1041-1043 (1924).

[59] Löhr, W., "Weitere Ergebnisse bei Anwendung der Blutkörperchensenkungsprobe in der Diagnostik chirurgischer Erkrankungen," *Deuts. med. Wochenschr.*, 48, 388-389 (1922).

[60] Witkower, E., "Die Veränderungen des Blutes bei der Anaphylaxie," *Z. ges. exper. Mediz.*, 34, 108-118 (1923).

of the red blood cells of citrated blood collected during anaphylactic serum shock in the guinea pig. I observed the same fact with M. La Barre for oxalated blood collected under the same conditions.

The sedimentation of the red cells is very much slower in citrated or oxalated blood taken from the carotid of dogs during anaphylactic serum shock. The red blood cells settle in citrated or oxalated blood of dogs and guinea pigs the slower the more marked the intensity of shock and the lowering of the surface tension of the plasma. The sedimentation of the red cells begins sometimes only after several hours, even when the carotid blood has preserved its arterial appearance, as is usually the case with the dog.

The red corpuscles settle more slowly than in the normal state during peptone shock in the dog;[61] however, this phenomenon is less marked than during the course of anaphylactic shock. After $\frac{1}{2}$ hour there is never any sedimentation during anaphylactic shock; it is usually the same in peptone shock, but in some cases there is some sedimentation at this period. After 2 hours it has hardly started in anaphylactic shock; at this moment it is generally very moderate in peptone shock, although it sometimes attains a value which is a little higher than the value observed before the injection of the serum into the experimental dog. The sedimentation of the blood cells after 24 hours is still slight in anaphylactic blood; it is markedly increased after the second hour in blood taken during peptone shock, and its intensity is sometimes close to $\frac{2}{3}$ or $\frac{4}{5}$ of the value observed in the same animal in a normal state. Table XII shows in per cent the values obtained with citrated blood obtained during first bleeding. the average values of sedimentation from the second bleeding in different portions of blood from normal dogs, or from animals during full anaphylactic or peptone shock.

TABLE XII.

Time (in Hours) After Which the Degree of Sedimentation Has Been Determined in Hours	Condition of Dogs After the Second Bleeding	Sedimentation of Blood Cells from Second Bleeding in per Cent of the Initial Values (Citrated Blood from First Bleeding)			
			Blood Diluted with $\frac{1}{10}$ of its Volume		
		Undiluted Blood	With NaCl 0.9 per Cent	Sodium Oxalate 1 per Cent NaCl 0.65 per Cent	Citrate of Sodium 3.55 per Cent
2	Normal	....	....	72.10	89.03
	Peptone shock .	23.95	18.80	24.71	28.72
24	Normal	....	....	82.22	90.99
	Peptone shock .	58.66	51.79	59.68	60.97
	Anaphyl shock .	13.35	14.78	10.49	20.20

The following facts are clearly evident from this table:

1. The velocity of the sedimentation of the erythrocytes of citrated or oxalated blood is somewhat lowered in the normal state during the first

[61] Zunz, E., "Relations entre la vitresse de sedimentation des hématies et la tension superficielle dans les états de choc.," *Compt. rend. Soc. Biol.*, **92**, 1119-1122 (1925); "A propos de la vitesse de sedimentation des hématies de chien," *Compt. rend. Soc. Biol.*, **94**, 1024-1028 (1926).

bleeding. The very considerable retardation during peptone and, particularly, during anaphylactic serum shock, depends certainly upon other factors.

2. The erythrocytes settle with about the same velocity in oxalated blood and in blood without anticoagulants; this is particularly marked during peptone shock.

3. During anaphylactic, or peptone shock, the sedimentation of red cells proceeds more quickly in the citrated medium than in the undilated plasma. The presence of citrate counteracts, at least under these conditions, the retardation of the sedimentation of the red cells.

4. The dilution by means of a sodium chloride solution diminishes the sedimentation of the red cells in blood, taken during peptone shock and does not seem to exert any real influence upon this phenomenon in blood collected during anaphylactic shock.

5. If the preceding data could be verified during other experimental conditions and in other species of animals, oxalated blood should be preferred to citrated blood for the determination of the velocity of the sedimentation of the red cells in their plasma.

Asphyxia usually does not at all modify the velocity of the sedimentation of the erythrocytes, but tends sometimes to accelerate it and slightly to diminish it in exceptional cases. These, at least, are the results with the carotid blood of guinea pigs collected during asphyxia 1½-3 minutes after the ligation of the trachea.

When saturating with oxygen asphyxiated blood or anaphylactic blood of venous appearance, the velocity of sedimentation of the erythrocytes is not modified. It is the same when carbon dioxide is allowed to bubble through carotid blood taken from normal guinea-pigs.

The sedimentation of erythrocytes is, according to Ritzenthaler,[62] very much delayed, or even sometimes completely inhibited, in blood, taken from the jugular shortly after reinjection with a maceration of beef, or of egg white in horses pretreated by means of these products. The diminution of the velocity of sedimentation of the erythrocytes seems thus to be a constant symptom during anaphylactic shock.

A retardation of the sedimentation of the red cells is also observed in citrated or oxalated blood, taken from unused guinea-pigs after the intravenous injection of homologous agar-treated serum, but this phenomenon is less marked than in anaphylactic shock. The globules settle slower the more violent the shock and the more lowered the surface tension of plasma. During the most violent shock, the velocity of sedimentation remains normal On the contrary, an acceleration in the velocity of sedimentation of erythrocytes is observed in shock, caused in the guinea-pig by intravenous injection of 0.5–1 cc. of electrarzol or of 0.25–0.75 of 10 per cent solution of Witte's peptone (per 250 grams of body weight), which coincides with a sometimes considerable lowering of the surface tension of plasma.

The lowering of the surface tension does not seem entirely to be due to exactly identical causes in the different shocks. This is probably the cause of the differences in the velocity of sedimentation of red cells under different conditions. The velocity of the sedimentation of the erythrocytes depends in fact upon numerous factors; the proportion between plasma and globules, the number of erythrocytes, the more or less marked agglutination, the density

[62] Ritzenthaler, M., "L'anaphylaxie du cheval," *Arch. int Physiol.*, 24, 54-72 (1924).

and viscosity of the plasma and of the whole blood, the fibrinogen content of plasma and perhaps also of sero-globulin, lecithin, salts, etc.

It is without doubt quite appropriate to point out the relation between the retardation of the sedimentation of the erythrocytes during anaphylactic shock, and the increased content of erythrocytes and the increase of the viscosity of the whole blood. The fibrinogen content of plasma does not seem to play any rôle because the sedimentation during anaphylactic serum shock in dogs is very much retarded even when the fibrinogen content is slightly increased. Or the increase of the fibrinogen and sero-globulin content of plasma is, in general, associated with a lowering of the surface tension of plasma, and an increase of the velocity of the surface tension of plasma and an acceleration of the velocity of sedimentation of the erythrocytes. This is at least the case in man, partly under the influence of intense and prolonged irradiation, and in part also in untreated syphilis with a positive Bordet-Wassermann reaction.

It is still impossible to determine at the present the causes of the modifications of the surface tension of the plasma and of the velocity of sedimentation of erythrocytes during the different kinds of shock, just as well as the reasons of the very divergent observations on the velocity of sedimentation of erythrocytes in guinea pigs and dogs, after intravenous injections of analogous proportions (with regard to the body weight) of a 10 per cent solution of Witte's peptone. I hope to take up this problem later.

IX. Influence of X-Rays upon the Surface Tension of Plasma

After massive irradiation with intensely penetrating X-rays, there has been observed a syndrome known as "the disease of penetrating irradiations," which has been considered by certain authors as due to hemoclasic shock. Govaerts, Sluys and Stoupel [63] have found a lowering of the surface tension of citrated plasma during such a condition in persons subjected to prolonged applications of X-rays of a considerable dose (1500–6000 R).

La Barre collected blood from different dogs before irradiation, then 2–24 hours after application of X-rays in doses differing in from 250-750 R.[64] During these experiments he could not detect any considerable changes of the surface tension of plasma. It showed normal values before (74.8–75.5 dynes per cm.) and after irradiation (74.5–75.4 dynes per cm.). Generally there is observed a very slight lowering after irradiation, but this is not always the case; and if a lowering exists, it is so minimal (0.1–0.3 dynes) that no real importance can be attributed to it. Now phenomena of marked hypecoagulability of the blood have already been observed under these conditions. These may appear already in the absence of all modifications of surface tension of plasma, and it is not at all certain that all the manifestations of "pain from penetrating irradiations" must be attributed to haemoclasic shock. Particularly, with regard to the hypercoagulability of the blood, it should be correlated with the hyperadrenalism observed under these conditions.[65]

X. Surface Tension of Human Plasma in Syphilis

Due to the kindness of different syphilologists, I was able to examine with Mr. La Barre [25] the oxalated plasma of 44 male syphilitic patients. We thus learned that the surface tension of plasma is lowered in untreated syphilis. This phenomenon is, as a rule, more marked during the tertiary stage than during the secondary stage, or particularly during the primary stage. It seems even to be more pronounced in hereditary syphilis, of which we could unfortunately examine but one case. It is, on the other hand, less marked in the nervous forms of syphilis (tabes, general paralysis) than in the other forms of this disease.

The surface tension of oxalated plasma is, on the average, lower when the Bordet-Wassermann reaction is positive (70.4 dynes per cm.) than when it is negative (71.9 dynes per cm.). It is, however, not quite possible to recognize an exact relationship between the average degree of the lowering of the surface tension of oxalated plasma and the intensity of the Bordet-Wassermann reaction. The surface tension of syphilitic plasma with an intense Bordet-Wassermann may still exceed the surface tension of a negative syphilitic plasma.

During treatment with arsenobenzol and mercury preparations, the surface tension of plasma may become normal. While the surface tension in untreated syphilitics is lower than water (73 dynes per cm.), it is never lower than that of water in treated patients, and may exceed it even by one or two dynes, so that the surface tension shows normal figures in most of these cases, however, remaining always somewhat lower than the average surface tension of oxalated normal human plasma.

The question arises, why is the surface tension of plasma lowered in untreated syphilis? One is inclined to explain this fact by an increased content of fibrinogen and seroglobins in the blood of syphilitic patients.[66] Under the influence of arsenobenzol the blood again shows its normal content of serumglobulins [67] and its plasma shows the tendency to become normal, while the Bordet-Wassermann reaction disappears. The fibrinogen and serumglobulin content increases in certain morbid conditions, while there is no corresponding lowering of the surface tension of plasma.

The quantitative variations of the different kinds of proteins in plasma do not explain sufficiently why the surface tension of syphilitic plasma shows lower values. It might perhaps be necessary to take account of the qualitative variations of these proteins.

Herzfeld, Hirschfeld and Klinger [68] have, on the other hand, reported some evidence which would make it probable that the plasma of syphilitics contains capillary active cleavage products of proteins.

[66] Noguchi, H., "The relation of protein, lipoids and salts to the Wassermann Reaction," *J. exper. Med.*, 11, 84-99 (1909). Winternitz, R., "Ein Beitrag zur chemischen Untersuchung des Blutes rezent luetischer Menschen," *Arch. Dermat. Syphil.*, 93, 66-72 (1908); "Zweiter Beitrag zur chemischen Untersuchung des Blutes rezent luetischer Menschen," *ibid.*, 101, 227-246 (1910). Bircher, M. E. and McFarland, A. R., "The globulin content of the blood serum in syphilis," *Arch. Dermat. Syphil.*, 5, 215-231 (1922).

[67] Noguchi, H., *loc. cit.*, Tokuda, I., "Refractometric studies in human syphilis with special changes during treatment with arsphenamin and neoarsphenamin," *Arch. Dermat. Syphil.*, 5, 512-525 (1921); "Refractometric studies with the serum of normal rabbits receiving intravenous injections of arsphenamin and neoarsphenamin," *ibid.*, 5, 616-621 (1921).

[68] Herzfeld, E. and Klinger, R., "Chemische Studien zur Physiologie und Pathologie," *Biochem. Z.*, 73, 42-61 (1917); 87, 36-76 (1918). Hirschfeld, L. and Klinger, R., "Über das Wesen der Inaktivierung und der Komplementbindung," *Z. Immunitätsf. exper. Therap.*, 21, 40-76 (1914); "Zur Frage der Cobragiftinaktivierung des Serums," *Biochem. Z.*, 70, 398-410 (1915).

There is also another factor to consider. Some clinicians admit that the liver is affected in syphilis at an early date. The biliary acids and biliary salts lower the surface tension of plasma considerably. The plasma in icterus (jaundice), whose blood contains not only bile pigments, but also more or less of bile salts, has a lowered surface plasma with an average value close to that of the plasma of untreated syphilitic patients. It would be of importance to determine exactly the content of biliary acids in plasma, in the normal state and in syphilis.

In any event, it would be rather premature to decide in favor of any of the different hypotheses advanced.

Numerous authors [69] have found that the velocity of sedimentation of the erythrocytes is increased in citrated plasma from syphilitic patients. According to Nathan and Herold,[70] there is a slightly increased velocity of sedimentation in primary syphilis with a negative Bordet-Wassermann reaction; it is much accelerated in primary syphilis with a positive serum reaction and particularly in secondary syphilis; the acceleration is considerably diminished in tertiary syphilis with a positive serum reaction; it is very slight in latent syphilis with a positive Bordet-Wassermann; the erythrocytes settle in a normal manner in latent syphilis with a negative serum reaction. The velocity of sedimentation of the erythrocytes is increased in congenital syphilis and in general paralysis. While György [71] admits a parallelism between the intensity of the Bordet-Wassermann reaction and the rapidity of the sedimentation of the erythrocytes, Nathan and Herold have a different point of view. These authors have in fact seen the serum reaction disappear in 4 patients before the velocity of sedimentation of the red cells had returned to the normal value.

I have observed, together with Mr. La Barre, that the erythrocytes settle more quickly in oxalated plasma of untreated syphilitics, than in normal plasma. Sometimes there is an acceleration in the oxalated plasma in tubes with a negative Bordet-Wassermann. The increase of the veolcity of sedimentation of the erythrocytes goes hand in hand in oxalated plasma of untreated syphilitics, with a drop in the surface tension.

The increase of the velocity of sedimentation of the red blood cells in untreated syphilitics, can be doubtlessly explained by the increased content in fibrinogen and serum globulin, as reported in 1909 by Noguchi and Winternitz,[66] with regard to untreated cases with a positive Bordet-Wassermann and verified repeatedly since by others.[72]

[69] Fåhraeus, R., *loc. cit.*, Plaut, F., *loc. cit.*, Runge, W., *loc. cit.*, Popper, E. and Wagner, R., "Über die Sedimentierungsgeschwindigkeit der Luetiker," *Med. Klin.*, 16, 922-924 (1920). Nadolny, G., "Über die Senkungsgeschwindigkeit der Blutkörperchen bei Säuglingen," *Berl. klin. Wochenschr.*, 58, 998-999 (1921). Mayr, J. K., "Die Sedimentierungsgeschwindigkeit der Blutkörperchen im citratblut," *Arch. Dermat. Syphl.*, 134, 225-231 (1921). Schönfeld, W., "Untersuchungen über die Sedimentierungsgeschwindigkeit des menschlichen Blutes unter besonderer Berücksichtigung des Blutes von Syphilitikern," *ibid.*, 136, 89-98 (1921).

[70] Nathan, E. and Herold, G., "Die Senkungsgeschwindigkeit der roten Blutkörperchen in den verschiedenen Stadien der Syphilis," *Berl. klin. Wochenschr.*, 58, 642-657 (1921).

[71] György, P., "Über die Senkungsgeschwindigkeit der roten Blutkörperchen im Säuglingsalter, im besonderen bei Lues congenita," *Münch. med. Wochenschr.*, 68, 808 (1921).

[72] Bircher, M. E. and McFarland, A. R., *loc. cit.*, Herzfeld, E. and Klinger, R., *loc. cit.*, Kapsenberg, G., "Untersuchungen über die Bedeutung der Globuline bei der Wassermannschen Reaktion zugleich ein Beitrag zur Technik der Dialyse und zur Ausführung der Wassermannschen Reaktion," *Z. Immunitätsf. exper. Therap.*, 31, 301-371 (1921). Kopaczewski, W., "Le mécanisme de la réaction de Bordet-Wassermann, *Compt. rend.*, 17, 1170-1172 (1920); "Les travaux récents sur le choc (1922-1923), *Gasette des hôpitaux*, 46, 1675-1680 (1923). Sachs, H., "Über Beziehungen zwischen physikalischchemischer Konstitution und Biologie des Blutserums," *Kolloid-Z.*, 24, 113-128 (1919).

XI. Surface Tension of Human Plasma in Other Morbid Conditions

Biliary acids and salts lower the surface tension of water and plasma considerably. Therefore, there is nothing astonishing in the more or less marked lowering of the surface tension of the plasma in icterus (jaundice), as shown by Kopaczewski [73, 74] in the plasma of icteric patients. We had the occasion to observe 7 light cases of icterus. The plasma surface tension was between 67.8–72.9; it averaged 70.8 dynes per cm., a figure identical with that obtained for non-treated syphilitics.

The surface tension of plasma remains normal in most of the diseases of the heart and circulatory systems, of the respiratory organs and the digestive tract. A slight lowering of the plasma surface tension is sometimes observed in infectious diseases with high temperatures, when it varies between the values of water and the lowest values obtained in the normal state (74.6–75.2) dynes per cm. It would be of importance to study a great number of tuberculous cases in order to find out whether the increase of the surface tension of serum, as announced by Kopaczewski, also occurs as often in plasma.

XII. Surface Tension of Human Serum in the Normal State

Numerous authors have determined the surface tension of blood in man. Unfortunately they often failed to take account of the density of this medium. This is the case for the stalagmometric determinations published by Kunoff,[75] by Kascher,[76] Kisch and Remertz,[77] Traube,[78] Boenheim.[79]

Iscovesco [80] reports the surface tension of 3 specimens of human serum as 69.40, 69.97 and 70.12 dynes per cm.* Kopaczewski considers as the normal value of the surface tension of normal human serum between 66–69 dynes, with an average value approaching 68 dynes per cm.** The densities vary between 1.0265 and 1.0325 and average 1.027.

It seemed to us necessary to compare the surface tensions of plasma and serum obtained from the blood specimens of the same persons, collected immediately one after the other without stasis from one of the veins in the bend of the elbow, during fasting, or after a light meal containing no fats or very little fat. With Mr. La Barre this procedure was carried out on 5 young men from 21–22 years of age. Having found slightly higher values for the

[73] Kopaczewski, W., "Les caractères physico-chimiques du sérum au point de vue de la réaction de Bordet-Wassermann," *Compt. rend. Soc. Biol.*, **82**, 1269-1271 (1919).
[74] Kopaczewski, W., "Sur les modifications physico-chimiques dans l'étiologie des états morbides," *Congr. Franç. de méd. int. Paris*, **2**, 287-291 (1922).
[75] Kunoff, R., Inaug.-Dissertation, Berlin, 1907. Quoted from Bottazzi, in Neuberg, C., *Der Harn*, Berlin, **2**, 1724 (1911).
[76] Kascher, S., "Die Oberflachenspannung von Körpersaften unter normallen und pathologischen Bedingungen," Inaug.-Diss., Berlin (1907).
[77] Kisch, B. and Remertz, O., "Über die Oberflächenspannung von Serum und Liquor cerebrospinalis beim Menschen und über die Technik kapillarimetrischer Messungen," *Münch. med Wochenschr.*, **41**, 1097-1099 (1914); "Kapillarimetrische Untersuchungen am Serum und Liquor cerebrospinalis des Menschen," *Int. Z. physikal.-chem. Biol.*, **1**, 354-388 (1914).
[78] Traube, J., "Physikalisch-chemische Untersuchungen von Blutseris," *Int. Z. physikal.-chem. Biol.*, **1**, 389-411 (1914).
[79] Böhnheim, F., "Die Oberflachenspannung des Mageninhalts sowie ihre Veranderung bei natürlichen und künstlichen Verdauungsversuchen," *Z. Biol.*, **94**, 174 bis 193 (1919).
[80] Iscovesco, H., "La tension superficielle du sérum sanguin," *Compt. rend. Soc. Biol.*, **70**, 66-67 (1911).
* The densities of these sera were very low: 1.019, 1.020 and 1.022. Iscovesco accepts 75 dynes as the surface tension of water. His stalagmometer presents many sources of error. These figures have, therefore, no real value.
** Kopaczewski calculates the surface tension by taking 73 dynes as the value for water at 15° C.

surface tension of serum than those obtained by Kopaczewski, we extended our experiments over a large number of normal individuals, by examining their serum. We studied altogether 20 young men from 19–24 years of age, one man 47 years of age, and 3 women aged 24, 35 and 40 years. These 24 persons were perfectly healthy with a negative Bordet-Wassermann. The different samples of blood were collected directly in centrifuging tubes and then submitted to the same manipulations as the samples of oxalated blood. They were centrifuged at high speed for about 2 hours in order to separate the sera and then centrifuged a second time for 20-30 minutes. The upper layer was then pipetted off and the density and the surface tension determined immediately afterwards.

We discarded all the sera which were not perfectly clear. Cloudy sera have, in fact, always a lower surface tension than clear sera, as has already been shown by Kisch and Remertz.[27] Iscovesco[4] and Kisch and Remertz have shown that traces of hemoglobin are sufficient appreciably to lower the surface tension of serum. We saw before that the same is true for plasma.

It is indispensable to separate the serum from the clot as soon as possible, because the surface tension of serum becomes gradually lower, when it remains in presence of a fibrin reticulum enveloping blood corpuscles, even when in the icebox. There are certain changes which result in capillary active substances (other than hemoglobin) entering the serum.

It is also not possible to preserve even a perfectly clear serum at room temperature before determining its surface tension. The latter becomes in fact gradually lower, doubtlessly due to autolytic phenomena causing the formation of capillary active cleavage products (proteoses, for example).

Considering only those values obtained with specimens of serum examined only a few hours after bleeding, and taking care to avoid the numerous sources of error to which we have just pointed out, the surface tension of normal human serum is between 69.1–70.6 dynes per cm. It averages 70 dynes, being 3 dynes less than water and 5 dynes below that of plasma. The dynamic surface tension of serum is remarkably constant for the human species, as is the case with other mammals heretofore examined. The average value of the surface tension of human serum is identical with that found by Cosmovici[16] for the dog and very close to that found by the same author for the rabbit (70.1 dynes per cm.).

By examining separately the two sexes we obtain a somewhat higher average value for women (70.2 dynes per cm.), than for men (69.9 dynes per cm.), as we have before observed it for plasma. But the very small number of sera from women so far examined, should make us very cautious in drawing conclusions in this respect. Numerous determinations of the surface tension of serum and plasma should be made before we will be able to state definitely what influence sex exerts upon the surface tension of plasma and serum.

XIII. Surface Tension of Human Serum in Syphilis

I could detect together with Mr. La Barre a slight lowering of the average surface tension of serum in untreated syphilis. This lowering of the serum and plasma is, in general, more marked in the tertiary stage than in the secondary and, particularly, than in the primary stage.

The surface tension of serum may present a normal value (even close to the average normal) in syphilis, in secondary syphilis and in tabes; this is not the case in tertiary syphilis. The surface tension of plasma is always lower in untreated syphilis, when compared with normal, and never reaches the value of the surface tension of water.

The serum has similarly to the plasma a lower average surface tension when the Bordet-Wassermann is positive (68.2 dynes per cm.) than when it is negative (68.8 dynes per cm.). But sometimes a lower surface tension is obtained with syphilitic serum with a negative Bordet-Wassermann than with that from a patient with an intense positive reaction. The average lowering of the surface tension of serum is not strictly parallel in its intensity with the Bordet-Wassermann reaction. The lowest average values for the surface tension have sometimes been observed with sera showing intense Bordet-Wassermann reactions.

The influence exerted by syphilis upon the changes of the surface tension of plasma and of serum, can be well understood, by comparing the results obtained with the two media in the same person during health, or during the different stages of syphilis. Light is thus thrown upon the constant and undeniable lowering of the surface tension of plasma in syphilis, while it is rarer (5–12 times) and in general less intense in the serum of the same patients.

These results do not agree entirely with those obtained by Kopaczewski. According to this author, the average surface tension of human serum is 67.5 dynes per cm. in the normal state, 69.5 dynes in syphilis. The surface tension of serum would therefore be markedly increased in syphilis, according to Kopaczewski.[72, 73, 74]

The researches of Elfer,[82] Kisch and Remertz,[27] Traube [78] do not disclose any appreciable changes of the surface tension of serum.[81]

The contradictions, which we have just referred to, are partly due to the fact that the serum separated from the clot has not been collected and examined soon enough. They are partly also due to continuous changes of the surface tension of serum, as has been so well pointed out by Lecomte du Noüy[28] and by Kopaczewski.[10]

XIV. The Advantages of Plasma Over Serum for the Determination of Surface Tension

Sachs and Oettingen [24] have already pointed out the advantage of plasma over serum for the determination of surface tension, by calling attention to the fact that notwithstanding the presence of anticoagulants, the plasma represents far better than serum the true blood medium, as it exists during life.

It results clearly from the investigations of Herzfeld, Hirschfeld and Klinger [68] and other authors, that the different kinds of serum proteins vary mainly in their physicochemical characteristics and that there exists a gradual transition between the very labile fibrinogen, the less labile globulins and the relatively stable albumins. Now, blood serum no longer contains the most

[81] Weill, O., "Les colloïdes en thérapeutique," notes sur les conférences du professeur Kopaczewski," *Bruxelles-Médical*, **4**, 397-408 (1924). Iscovesco, H., "L'influence de l'hémoglobine sur la tension superficielle," *Compt. rend. Soc. Biol.*, **70**, 11-12 (1911).
[82] Elfer, A., "Über einige Eigenschaften des syphilitischen Blutserums vom immuno-chemischen Standpunkte," *Folia Serologica*, **3**, 461-470 (1909).

labile blood protein constituent, the one most subject to physicochemical changes. There is, therefore, nothing surprising in the fact, that the physicochemical changes of the blood can be better observed in the plasma than in the serum.

An increase of the fibrinogen content has been stated [83] to occur in pregnancy and in different infectious diseases (pneumonia, pulmonary tuberculosis, pleurisy, peritonitis, rheumatic fever, erysipelas, scarlet fever). There is simultaneously also an increase in the content of serum globulins. [84]

The determination of the surface tension seems certainly to be subjected to a greater number of errors in properly collected serum than in the plasma.

Our investigation supports entirely the viewpoints of Sachs and von Oettingen. We recall that almost identical values are obtained for citrated, hirudinized and oxalated plasma taken from the same person. The dilution of the plasma by adding $\frac{1}{10}$ of its volume of one or the other anticoagulating solutions, does not seem to modify its surface tension appreciably.

The oxalated physiological solution used in man had a density of 1.015 and a surface tension of 74.7 dynes per cm. at 18° C. The citrated solution had a density of 1.023 and a surface tension of 73.3 dynes per cm. at 18° C.

We believe that oxalated plasma should be used in the future, whenever possible, for the determination of the surface tension of blood. In this way more constant results will be obtained and more marked differences will be found when using serum; but it is necessary thoroughly to mix the oxalate with the plasma, in order to avoid all traces of coagulation, which would lower the surface tension to a variable degree, to collect the plasma during fasting or after a light meal not containing any fat, and to discard all plasma which is not perfectly clear, or which contains hemoglobin. It is, besides, much easier to obtain a plasma than a serum free from blood pigments. We cannot sufficiently emphasize the necessity of determining the surface tension only in plasma or in serum in a perfect condition, and the considerable advantages which plasma presents in this respect.

In this way only the investigations carried out on plasma will allow us, without doubt, to throw light upon the rôle which surface tension plays in the action of general anesthetics and in many other problems of phamacodynamics and pathology.

We may at present state that a lowering of the surface tension of plasma is seen in the rabbit and the dog 10–20 minutes after intravenous injection of large doses of amytol, chloralose and urethane, but this phenomenon becomes

[83] Halliburton, W., "A text-book of chemical Physiology and Pathology," Lond., 306, 1891. Lackschewitz, "Über die Wasseraufnahmefahigkeit der roten Blutkörperchen nebst einigen Analysen Pathologischen Blutes," Inaug.-Diss., Dorpat, 1892. Pfeiffer, T., "Über den Fibringehalt des menschlichen Blutes und die Beziehungen desselben zur sog. Crusta phlogistica," *Z. klin. Med.*, 33, 215-257 (1877). Berggrun, E., Über Fibrinausscheidung beim gesunden und kranken Kinde nebst Analysen normalen und pathologischen Blutes," *Arch. Kinderheilk.*, 18, 178-179 (1897). Lewinski, J., Beobachtungen uber den Gehalt des Blutplasmas an Serum albumin, Serumglobulin und Fibrinogen," *Arch. ges. Physiol.*, 100, 611-634 (1903). Landsberg, E., Untersuchungen über den Gehalt des Blutplasmas an Gesamteiweiss, Fibrinogen und Reststickstoff bei Schwangeren," Inaug.-Diss., Halle (1912). Krösing, E., "Das Fibrinogen im Blute von normalen Graviden, Wochnerinnen und Eklamptischen," *ibid.*, 94, 317-331 (1911). Dochez, A. R., "Coagulation time of the blood in lobar pneumonia," *J. exper. Med.*, 16, 693-700 (1912).

[84] Halliburton, W., *loc. cit.*, Limbeck, R. v. and Pick, F., "Über die quantitativen Verhältnisse der Eiweisskörper im Blutserum von Kranken," *Prag. med. Wochenschr.* (1893). See also *Maly's Jahrb. f. Tierchemie*, 23, 162. Epstein, A. A., "A contribution to the study of the chemistry of blood serum," *J. exper. Med.*, 16, 719-731 (1912). Epstein, A. A., "Further study of the chemistry of blood serum," *ibid.*, 17, 444-452 (1912). Rohrer, F., "Bestimmung des Mischungsverhältnisses von Albumin und Globulin im Blutserum," *Deut. Arch. klin. Med.*, 121, 221-240 (1916). Alder, A., "Anhaltspunkte für die Prognosenstellung der Lungentuberkulose aus refraktometrischen und viscosimetrischen Serumuntersuchungen," *Z. Tuberkulose*, 31, 10-17 (1919).

rapidly weaker and will even sometimes disappear entirely ½ hour after the introduction of the hypnotic into the circulation, while the animal is in profound anesthesia and awakens only a few hours later. The blood taken during narcosis by inhalation of chloroform or ether furnishes almost always a plasma with an absolutely normal or very slightly lowered surface tension. This slight lowering sometimes seen during prolonged narcosis from inhalation of chloroform or ether seems to be caused by the presence of biliary acids and other capillary active products in the blood.

The Colloidal State in Living Organisms

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A fact of considerable importance confronts us when we consider living matter, a fact supported by such evidence that no biologist has ever questioned it. We refer to the fact that the substances which enter into the constitution of living matter exist, for the most part, in colloidal condition.

All cells, from the simplest protophyte to those which form metazoans highest in the scale of the evolution of species, appear in the colloidal state. The cell membranes, the protoplasm, the nuclei of these cells, are all colloids. The fluids of cellular origin which perfuse plants, as well as the fluids which circulate in animals or which bathe their tissues, likewise exhibit this same structure. Life cannot exist apart from this particular form of matter, and we may, in brief, say that the colloidal state is the basis of life.

We know, furthermore, that colloids have special properties which distinguish them from all other substances; therefore it is unquestionable that these properties should be found in the basic substances of animals and plants which are colloidal in nature. Consequently we are surprised that so obvious a truth has not until recently attracted the attention of physiologists, though it must form the basis of all vital phenomena.

What are, then, the main, specific and fundamental properties of colloid which take part in vital manifestations?

To define them, we should consider two main groups of facts: the spontaneous evolution of colloids, independent of all external interference, and the mechanism according to which these colloids react when they are brought into contact with agents capable of modifying them.

It is unnecessary to dilate upon the constitution of colloids; on their structure, formed of complex micells, including granular, polymolecular, and insoluble masses; surrounded by perigranular substances, which they hold by adsorption, these micellular elements carrying electric charges and actuated by Brownian motion: this constitution is well known, and has been often described. What is most important to realize, is the continuous and fatal evolution of colloids, which are in constant transformation.

The particles of these colloids, driven by the molecular agitation of the fluids surrounding them, dash against each other constantly. When, in the course of their encounters, the forces which drive them together balance and exceed the repulsion arising from their electric charges, the granules unite and cohesion henceforth holds them together., The groups of particles grow gradually and when they have reached sufficient size, they can no longer remain in suspension and they precipitate; flocculation occurs, and the colloidal state is destroyed.*

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* Translated by Jerome Alexander.
* Here M. Lumière obviously means that the *sol* state of the colloid is destroyed, for as a rule, with biocolloids, flocculation means the transformation of a sol into a gel. *J. A.*

This phenomenon, known under the name of ripening or maturation, is inevitable. All colloids have then a span of individual existence and an age at any given moment; they age and their original structure is finally destroyed. The duration of this transformation is extremely variable, depending on the viscosity of the intermicellular fluid, the composition and form of the granules, external chemical and physical influences.

Independent of these evolutionary changes, another property of matter in the colloidal state plays an important rôle in biological phenomena; that is, the disparity between the effects of destructive reagents. and the percentages in which they are effective. To visualize this curious property, let us take a concrete example.

Consider a ferric oxide sol, properly dialyzed, in which for instance one kilogram of this oxide is maintained in colloidal condition because of the presence of a gram of ferric chloride in the perigranular substance, which is adsorbed by the particles, and is indispensible for maintaining in suspension a thousand times its weight of oxide.*

In this colloid, the iron oxide is insoluble and inert. although ferric chloride reacts readily with various reagents, with alkalis, for example. Quite a few substances when added to the colloid, combine with its ferric chloride and destroy its colloidal state; thus 0.30 gram of ammonia suffices to flocculate the entire mass of ferric oxide.

The reaction does not, then, proceed in this case as in the case of true chemical phenomena, where the effects are proportional to the weights of the reactive substances involved: minute quantities of substance are able to accomplish the precipitation of considerable quantities of colloid.

Starting from these very simple premises—colloidal ripening and inevitable evolution toward flocculation, and the disparity between effect and amount of reagent—we will be able to throw much light on a large number of biological and pathological facts which have heretofore remained inscrutable.

Variation of Nutrition and Growth with Age

At the beginning of the life of an individual, the colloids of the cells that compose his organs, have just been formed, the constituent micells are of minimal size, and then they gradually grow with time. Now the rapidity of the organism's growth necessarily depends upon nutritional activity, that is, the interchanges which take place within the organism; and this very activity depends on the external micellular surface, which increases with the degree of dispersion of the particles.

As particles increase in size by aggregation, their surfaces diminish correspondingly, and this parallels interchange. Nutrition and growth are very active in young organisms, and gradually slow up with age, because with micellular aggregation, the intensity of interchange and of nutrition progressively fall off.

The Problem of Immortality

Since the transformation of colloids moves always in one direction without ever returning to a prior condition, so that they ripen and in the end always flocculate, all beings are destined to perish. The irreversibility of

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* Sorum has just reported the production of ferric hydroxide sols entirely free of detectable traces of Cl. *J. A.*

these phenomena, which terminate in death, is a consequence of this fundamental property of colloids to develop fatally towards flocculation.

It has been supposed that certain protozoans, which, without conjugation, produce thousands of generations, do not die, but that in reality, in the course of their multiplication, they periodically eliminate nuclear and protoplasmic substances which correspond to the coagulation of ripened colloids. Protozoans thus die piece-meal, a phenomenon known under the name of *endomixia*. Furthermore, since matter is not infinitely divisible, the fourth generation can not have a single atom of the original ancestor.

SENILITY

Senility results from the invasion of organs by connective tissue, and this process is readily explicable if we remember that the epithelial cells have a protoplasm whose colloids are relatively fluid and consequently ripen more quickly than those of connective tissue cells, which are more viscous and which accordingly transform more slowly. Epithelial elements therefore cease growing and disappear by flocculation, while connective tissue elements continue to live and develop.

There is no doubt that certain poisons permeate the body economy, and, as is the case particularly with bacterial poisons of intestinal origin, accelerate colloidal ripening, preferentially affecting the more sensitive epithelial cells for the reasons above indicated, while the connective tissue cells continue to proliferate.

Finally it is through variations in the course of colloidal ripening that we may understand the differences in the time of gestation, growth, and longevity observed as we pass from one animal species to another.

PERIOD OF INCUBATION

Before the author explained the reason, no valid explanation of the incubation period of certain diseases had been proposed. True, in the case of infectious diseases it had been suggested that there was a rapid increase in microbes which finally were able to attack the defenses of the organism, but this puerile notion did not suffice to account for the sudden onset of pathologic symptoms, and still less for the necessity of an incubation period for a non-bacterial disease such as serum sickness or the symptoms occurring seven days after the administration of arsenobenzol.

Our colloidal theory furnishes the key to this enigma: the incubation period corresponds to the time necessary to ripen certain colloids, when they are brought under the influence of serum, chemical, or bacterial reagents, or else saturation with certain intermicellular substances.

The same phenomena may be reproduced *in vitro* with synthetic colloids. It is only when the intermicellular films are saturated or when the ripening reaches its limit, that flocculation follows suddenly, bringing with it a whole train of pathological difficulties.

DISPARITY BETWEEN EFFECT AND DOSE

Among the most obscure facts that biologists have thus far faced, we may mention those involving the startling effects that may be produced by minute

doses of certain poisons or certain medicaments. Bacterial products, viruses, venins, certain therapeutic agents, etc., possess extraordinary activity.* These substances do not act in proportion to their weights, as do definite chemical agents, and it is for this reason that they have been termed *sensitizers;* but this designation is merely a word that explains nothing.

It is easy to throw light on these phenomena by means of the property of colloids previously referred to: it is the intermicellular substance that enters into the reaction with the chemical substance, and as we have already seen, this substance represents but a very small fraction of the micellular complex. Besides there is a great diversity of colloids in the organism, each cellular group having its individual constitution; and furthermore the total weight of one of these cellular groups functioning specifically in an individual, may be very small. Take as a definite case, the sensory nerve cells of the first cervical pair. Granting that the cells of this group act as multiple colloids, each cell representing but a fraction of the total weight of the group, and of each one of these colloidal elements it is only the tiny intermicellular envelope that must enter into the reaction, we can understand how an infinite small amount of a substance may selectively combine with some one of the extremely small micellular fractions, and produce a partial flocculation which is able to disturb the functioning of the specialized cells, occasioning, in the case just cited, facial neuralgia.*

Symptoms Common to the Most Divergent Affections

Is it not remarkable that neither physiologists nor physicians have heretofore taken note of the eminently curious fact that diseases produced by the most diverse pathogenic agents show the same symptoms—all the more so because for this fact they have given no explanation.

A susceptible person comes into contact with a case of scarlet fever and is infected; another, being sensitive, is treated intravenously with arsenobenzol; a third, sensitized by a previous inocculation, receives a subcutaneous injection of horse serum: after an incubation of several days we see each one of these patients exhibit fever, an eruption, arthritis, etc.

Following rather differing courses, the symptoms of acute diseases always exhibit the same general common character: fever, chills, convulsions, vomiting, diarrhea, dyspnea, inflammation, pain, eruptions, etc., no matter what the causative agent.

Our colloidal theory of life and disease enables us readily to understand this community of symptoms. All these pathogenic agents, however they may differ, have one property in common: they determine the flocculation of certain humoral colloids whose coagulation produces the pathological symptoms. Furthermore, if we introduce experimentally into the circulation inert precipitates in suitable physical state, we can reproduce the symptoms of acute diseases: pruritis, fever, inflammation, vomiting, diarrhea, dyspnea, etc.

We do not yet know the exact form or the quantity of precipitate, whether

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* A. Krogh has shown that the posterior pituitary secretion contains a hormone effective in the proportion of about one part in a hundred million. John Abel purified this hormone until it was effective in 1 part in 18,750,000,000. *J. A.*

* A. Krogh found that the posterior pituitary hormone affects the tonus of the tiny Rouget cells surrounding the capillaries and regulating the flow of blood through them. See "The Anatomy and Physiology of Capillaries," Silliman Lectures for 1922 (Yale University Press). *J. A.*

flocculated or not, which is able selectively to bring about this or that symptom, but it is probable that some day we will be able to solve this problem.

MECHANISM OF PATHOLOGICAL SYMPTOMS

The basic changes underlying pathological symptoms remained profoundly mysterious until they were traced to flocculation. When an antigen enters the organism, be it an excretory product of a pathogenic bacillus, a toxic protein, or a poison of definite composition, it produces, according to its specific action and chemical affinities, the flocculation or precipitation of cellular or humoral colloids.

Humoral flocculates or precipitates, arising from the destruction of the colloidal state, excite the endothelial nerve ends of the vessels within which they circulate; by this mechanical excitation, they unbalance the vagosympathetic equilibrium, producing vaso-motor disturbances and more or less marked changes in action of the glandular system, the smooth muscular system, the sphincters, and especially of the erector pili muscles. Irritating the brain or bulbular centers, they cause nervous, respiratory, thermal consequences, etc.

According to their form and their dimensions and also to the region to which they are confined, they act as more or less effective impediments to the circulation of blood through the tissues. If their dimensions are very small, they reach the tiny capillaries of the skin and cause a dermatitis whose type varies with the physical condition and the amount of the flocculate or precipitate; in the lungs, they produce, through the sympathetic system, greater or less changes in the behavior of the smooth muscles which enter into play in the alveolae, and thus cause various vaso-motor disturbances. Thus arise atelectosis, congestion, and the different degrees of red hepatization.

If the dimensions of the flocculates are still larger, they are retained by vessels of still larger lumen, diminish the activity of metabolism and cause disturbances in the nutrition of tissues, whose importance is governed by the vascular permeability existing and the extent of the area attacked.

This view permits us to understand not only the origin of morbid symptoms but also the course of different diseases.

General disturbances of sudden onset, common to a large number of acute diseases, are caused by serum precipitations, the flocculation being, in this case, abrupt. The flocculates formed excite the endovascular sympathetic nerve ends, and especially the plexuses, and cause, at distant points, as our experiments have proven,[1] vaso-motor disturbances, visceral congestion, variation in the glandular secretions,[*] and in the functioning of the smooth muscular system; the action of the pulmonary alveolae is disturbed, parenchymatous hemorrhages are produced, and profound changes in the irrigation of the tissues and in the osmotic currents between the intra- and extra-vascular fluids arise, which modify local metabolism. When flocculates finally settle down in a certain area, they disturb the function of the organ in which they

[1] Lumière, A. "Le problème de l'anaphylaxie-Doin," Paris, 1914.
[*] In the case of the glands which produce internal secretions (endocrines) e.g. thyroid, suprarenals, pituitary, etc., the effects must be very marked. Thus a very tiny disturbance could activate or depress one of the endocrine glands, shifting the balance of the endocrine secretions, and resulting in a bodily change far out of proportion to the original disturbance—practically a trigger action. *J. A.*

deposit, determining localization, preparing the way for infection, the leuco-cytic defense being baffled either by circulatory insufficiency, or by the fact that the macrophages exert their action on the serum precipitate.

In chronic diseases, which proceed by crises separated by remissions of greater or less duration, serum flocculations are also produced, follow-ing the incidental successive invasions of antigen in a patient previously sensitized.

That the course of action is as above indicated, is proven by the fact that the experimental introduction, into the circulation of animals, of flocculates or of inert and insoluble precipitates incapable of chemical reaction, provokes most of these pathologic symptoms: by this means we can, at will, cause circu-latory and respiratory disturbances, convulsive crises, uneasiness, intense pru-ritis, fever, vomiting, diarrhea, fluid-pressure reactions, with the splanchnic organs, hemorrhages, sphincter reflexes, pulmonary atelectasis, or on the con-trary extreme alveolar dilation, red hepatization, cachexia, etc.

If we consider, on the one hand, that the excretory proteins of patho-genic bacteria invariably produce flocculates in the serum of sensitive subjects, and, on the other hand, that the introduction into the circulation of any sub-stance whatever in an insoluble state and deprived of all chemical reactivity, occasions the disturbances of acute and chronic diseases, we are naturally led to conclude that it is these precipitates which, by their physical excitation, cause the symptoms of these diseases.

This action manifests itself principally through the vago-sympathetic sys-tem, which governs all the phenomena of the vegetative or involuntary life. All the factors controlling involuntary or vegetative behavior are disturbed by the presence, in the humoral circulating fluids, of abnormal irritating sub-stances.

Apart from the possibility of reproducing these polymorphic pathological conditions with the aid of inert precipitates, we may borrow from clinical and experimental experience an argument which seems to us to give irrefutable proof of this conception.

Suppose we perform a phlebotomy on an epileptic, collecting the blood aseptically, and when the serum has separated from the clot, inject the serum into the circulation of a guinea-pig. Most of these sera are toxic, whereas the serum of a normal person is harmless under the same conditions. Certain specimens, from patients suffering from epilepsy, cause myoclonic convulsions in the guinea-pig, while others give rise to characteristic epileptiform seizures which may sometimes even cause the death of the animal.

According to the time when the serum is drawn from the patient (whether before, after or far removed from the seizures), and according to the form and gravity of the affection, the toxicity of the serum varies, but if we con-sider the most toxic sera we are forced to the conclusion that they carry the substance responsible for the seizures, because when injected they repro-duce the symptoms of epilepsy.

If, now, we prepare a guinea-pig by first carefully injecting into its vessels a small amount of a suitable prepared suspension of barium sulfate, to accustom the nerve ends of the centers, to excitation by flocculates, we can during the succeeding few hours, introduce with impunity into the circula-tion, a dose of epileptic serum which would be fatal without this precaution, and in this case, if we have done our work well, there will not be the least

sign of a seizure. The toxicity of the serum thus disappears completely, which proves that it depends on the physical action of a flocculate. Besides, the same serum freed from all precipitate by energetic centrifugation, by filtration through a bougie, or by prolonged standing, loses its convulsion-producing properties.

Furthermore, pathogenic substances which penetrate into the bodily interior may react selectively with the cellular colloids, and flocculate them.

When the protoplasm of nervous tissue is stricken by flocculation, we see neuroses or paralyses, according as the sensory or the motor cells are affected.

The cells are better protected than the humoral fluids because poisons at first meet the fluids which impregnate the organism, and can foil their precipitating action before they can reach the cells, whose protoplasm is still further isolated in the midst of its surrounding membranes. Therefore the general symptoms common to a great many diseases are of more frequent occurrence than the disturbances due to cellular flocculation. The transitory character of the former is comprehensible because no specialized essential bodily element is influenced by humoral flocculation, whereas if the specific proteins composing the cellular protoplasm are changed, the highly specialized and localized functions of the flocculated cellular elements, lead to much more lasting and often definite pathological manifestations, according as the cell can or can not repair the damage or be replaced.

ANAPHYLAXIS

It is the colloidal theory again which throws some light on the problem of anaphylaxis, which will remain an enigma unless there is taken into account the explanation we have given of this phenomenon, which will here be repeated.

When an animal is injected with a foreign protein, its humoral fluids develop the property of flocculating after a second injection of the same substance, made after the lapse of a certain time. The animal is thus specifically sensitized.

Upon reaching the brain centers, the flocculate excites the nerve ends of the capillary endothelium, which dilates; and this vaso-motor effect is transmitted by the sympathetic reflex system to the abdominal vessels which dilate in turn. The volume of the circulation is thus suddenly increased, while the mass of the blood remains unchanged; and there consequently results a sudden drop in arterial pressure which occasions the disaster known as *anaphylactic shock.*

The cause of this shock is, then, the flocculate which profoundly disturbs the vago-sympathetic equilibrium.

We have demonstrated the physical value of this process by a crucial experiment; we have shown that animals may be protected for several hours against the consequences of an activating dose, as well as against all anaphylactic shock, by carefully injecting into the circulation an inert substance, for example barium sulfate, which accustoms the interior walls of the vessels to irritation by flocculates.

All the experiments reported in recent years on anti-anaphylaxis by choline, acetylcholine, lipoids, colloidal metals, sodium oleate, thionine, nigrosine, peptone, etc., are only variations of our experiment, and a large number of sub-

stances may be thought of as being able to produce the same protective effect. They must be in a suitable physical condition, or else must form a precipitate with blood plasma.

The theory we have given regarding anaphylaxis can explain all the apparently paradoxical facts involved, which without its aid would remain altogether obscure, especially protection against shock [2] by ligature of the carotids; [3] by vaso-constriction, bleeding, or influence of decreased barometric pressure; [4] immunity of females during gestation, etc.

Chronic Diseases and Anaphylaxis

Thanks to our conception which refers pathological difficulties to flocculation, we can explain the course of development of certain chronic affections.

The internal structure of man and animals is normally protected against the penetration of proteins, the only substances which generally produce anaphylactic sensitization. The large molecules comprising these proteins can not pass through the skin, the epithelial walls of the digestive tract, or of the other mucous surfaces of the organism. These walls are, in fact, formed of cells in close juxtaposition and staggered in superimposed layers, thus forming a filtering barrier impenetrable by protein substances. However, an erosion, a trauma, an inflammation, may cause the epithelial barrier to lose its continuity, and foreign alimentary or bacterial proteins may then pass into the organism and sensitize it.

Upon a new change in the epithelial filter, when the same proteins come into contact with the body fluids of the subject already previously impregnated with them, they produce humoral precipitates and flocculations which do not in this case lead to the incidence of acute shock, but instead, to the manifold symptoms to which we have above referred, because the precipitates act more slowly.

The older the subject becomes, the greater the number of sensibilizations he suffers, so that his body fluids acquire increasingly complex reactive properties, and in growing old he is more and more subject to react to protein impregnations.

These phenomena are the basis of the explanation of chronic non-infectious diseases, and this harmonizes with the course of these affections which proceed in successive stages, and also with the fact that they have a greater incidence among the aged and may be cured by desensitizing.

Rôle of Normal Flocculates

In the course of metabolism and cellular multiplication, and during the functioning of the organs and the life of the tissue, the colloids composing living organisms are in a state of constant evolution. It must as a matter of course happen that some of them reach their ripening stage and are destroyed. There must therefore be continual normal flocculations, to as small extent, within the organism. Flocculated granules do not seem to accumulate

[2] Lumière, A., "La rôle des colloïdes chez las êtres vivantes," Masson, Paris, 1921.
[3] Lumière, A. and Couturier, H., "Dépression barométrique et choc anaphylactique," *Compt. rend.*, p. 1019, April 9, 1923.
[4] Lumière, A. and Couturier, H., "Résistance des femmes en gestation aux chocs anaphylactiques et anaphylactoïdes," *Compt. rend.*, p. 495, Feb. 13, 1922.

in the organism, and they must be destroyed and eliminated as fast as they are produced. It is probable that the precipitates thus formed are either digested by leucocytes, or else are eliminated by diffusion.

There thus occurs a balance which may vary somewhat with course of nutrition, digestion, and external controlling conditions, such as rest or exercise, surrounding temperature, etc.

Even during the periods of maximum normal flocculation, if the vascular system remains intact, the trivial precipitate in the circulation does not cause any difficulty; but if certain sections of the system lose their elasticity, as in sclerosis for example, especially in the nerve centers, or because of trauma or prior lesions, then disturbances may occur, especially when the blood is particularly rich in flocculates. It is probable that pathogenesis of certain psychopathic conditions, in diabetes and in Jacksonian epilepsy, may be due to this cause.

Will it not be possible to work out a plausible explanation of the alternation of sleep and waking from variations in excitation or in habituation of the nerve centers resulting from a periodic variation in the amount of flocculates distributed by the circulatory current?

The waking state results, no doubt, from an excitation of the brain centers, probably maintained by normal flocculates suspended in the blood; if the activity of the individual involves the disappearance of these substances, they no longer exercise the central irritative action which is necessary to maintain the waking state.

But this is purely an hypothesis which we mention with all reserve; whereas the other matters before referred to, regarding the mechanism of anaphylaxis as well as the rôle of flocculates in pathology, are, on the contrary, based on exact experiments, and we regard them as established.

TOXICITY OF TISSUE EXTRACTS AND TRAUMATIC SHOCK

We have shown [5] that the toxicity of tissue extracts and autolysates is consequent upon the fact that, on mixing cellular and humoral colloids, which are normally separated from each other by the membranes surrounding the cells, there is produced a flocculation. The mixture gradually becomes turbid and we have proven that it is the precipitate that causes the toxic symptoms caused by the injection of these extracts.

To this same mechanism must undoubtedly be attributed acute traumatic shock.[6]

The few headings under which we have just considered a number of physiological and pathological problems for which we have given an explanation, although they were previously considered to be shrouded in complete mystery, indicate the far-reaching disease.

This theory opens the widest horizon; I do not think that its correctness can be questioned, and so far I have found no facts incompatible with it. Quite to the contrary, whenever I have made experiments based on this doctrine, the results always confirmed my predictions. In my long experience as a biologist, I have for more than 30 years put it to innumerable tests in

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[5] Lumière, A., 'Toxicité des autolysates et des extraits tissulaires," *Compt. rend.*, **May 14, 1923.**
[6] Lumière, A. and Couturier, H., "Sur les chocs traumatiques," *Compt. rend.*, p. 776, **March 13, 1922.**

carrying out researches which were certainly not based on the new ideas that I have just published. These may be summed up as follows:

The colloidal condition controls life; flocculation is the mainspring of the sympathetic phenomena of subconscious life, and this flocculation it is that determines pathologic conditions and death.*

* The importance of protective substances and normal pII (about 7.35) in opposing flocculation, is obvious. *J. A.*

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Similarities in Colloidal Reactions of Blood, Simple Colloidal Solutions and Protoplasm

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In the discussion of the possible physiological action of blood, which is in part a colloidal dispersion, it is important, as Bayliss says, to keep in mind that the purely chemical behavior of those aggregations of molecules known as colloidal particles, must be a very inert one compared with that of the same material in true solution; for in the latter, all the molecules have the opportunity to react with any of the other molecules in the solution, whereas the colloidal particles are susceptible to action only on their surfaces. So that physiological action of serum on tissue would be dependent on the physico-chemical characteristics of the serum, e.g., the number of colloidal particles as seen with the dark field microscope, the surface tension, conductivity, membrane potential difference, H-ion concentration.

Blood serum observed with the dark field microscope, is distinctly a colloidal dispersion, its appearance varying with the character of the food and with the state of health. The variations in the number of visible particles have certain correspondences with the results of other methods of examination, which, in our laboratory, have formed themselves into a general scheme which follows (Fig. 1). It will be noticed that at the two extremes of pH 7.2 and 7.6 there are points of aggregation, while in the centre is what might be termed ultimate dispersion.

This question of the relation of aggregation and dispersion to pH is important, for here we would seem to have formed under certain conditions in living individuals a curve with two points of maximum aggregation in the serum. This produces the same shaped curve that Farr determined at Woods Hole when studying the maximum growth of root hair, which he said occurred at two different pHs; Hopkins also found the maximum rate of locomotion in amoeba to be at two points of about pH 6.5 and pH 8.0. However, in the aggregation and dispersion of colloidal particles in the blood serum, our experiments would seem to indicate that hydration and charge were more important than pH.

For this study 0.2 cc. of blood serum was always used. The serum was obtained from insane subjects. The serum was a constant, while the reagent
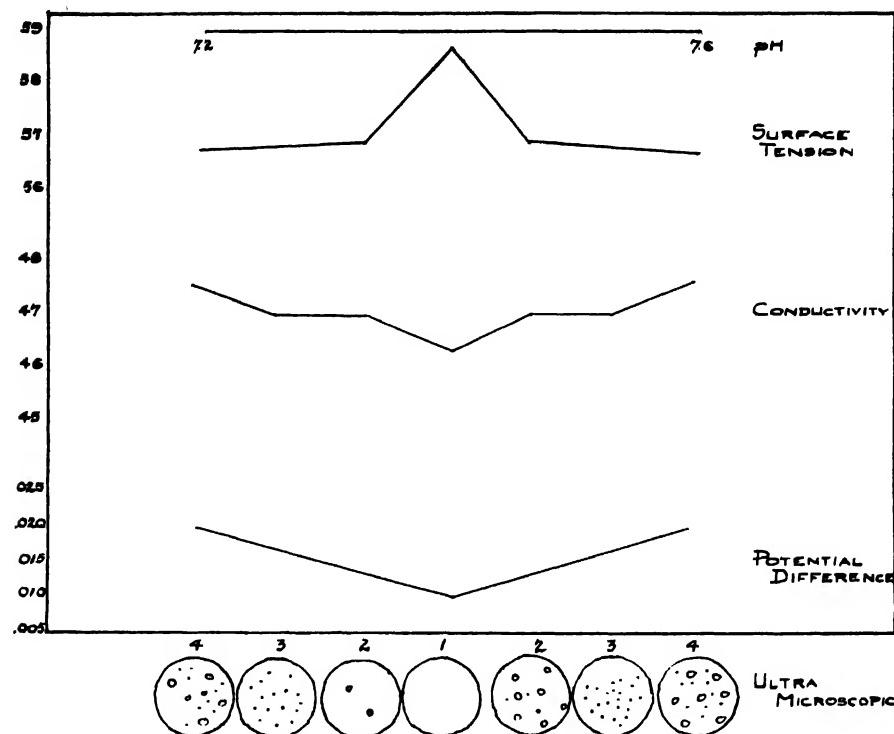
FIG. 1.—Variations in properties of blood serum with changing H-ion concentration.

used was in a series of concentrations. The effect of acid and alkali (HCl and NaOH) in aggregating the colloid particles was shown to occur in the following curve (Fig. 2):

This curve is similar to those demonstrated by Freundlich [1] with a Pt sol and FeCl$_3$, and by Kruyt [2] with a negatively charged mastic solution and AlCl$_3$.
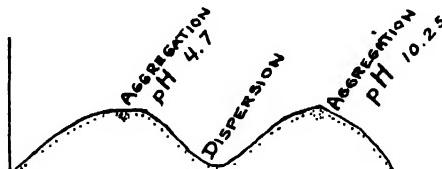
FIG. 2.—Diagram showing the behavior of the serum of insane patients upon addition of HCl and of NaOH.

Further experiment was made with various electrolytes in a series of concentrations. Serum and other electrolyte solutions were used in same relative proportions as with acids and alkalis.

KCl and Na-citrate gave practically identical curves, as shown in Figure 3. While in the case of the acid and alkali series the necessary range of pH

[1] Freundlich, H., "Colloid and Capillary Chemistry."
[2] Kruyt, "Colloids."

makes that appear as a determining factor, we obtained an almost identical curve with these salts, within a very limited range of pH. This would indicate, as demonstrated by Kruyt,[3] that the influence of the cation is the active factor, whether it is hydrogen or some other, although H— and OH— are the most active ions.
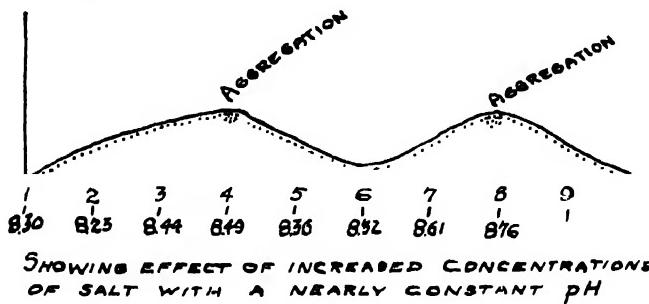
FIG. 3.

The effect of valence was demonstrated with $CaCl_2$, and Figure 4 indicates the results obtained.

In the given series of dilutions, three points of aggregation appeared with the bivalent cation, as compared with only two points of aggregation shown in the previous curve, which represents the effect of monovalency. This is in keeping with the rule that the action of ions is in proportion to their valence.
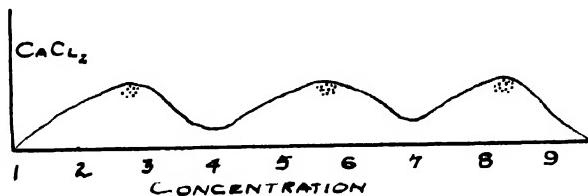
FIG. 4.– Diagram showing the effect of increasing addition of $CaCl_2$ on the blood serum of insane patients.

These curves fall in line with what has been termed "the irregular series", referred to by Freundlich (p. 429) and again by Kruyt (p. 85-86).* The latter found that in working with a negatively charged sol of mastic, small concentrations of $AlCl_3$ caused aggregation; slightly greater concentration produced a stable sol, and still further concentration resulted in another flocculation. In these series of varying zones of flocculation with non-flocculation the particles have also varying electrical charges. Such zones are said to occur whenever the potential lowering effect of the cation is far in excess of the potential-raising effect of the anion.

Since the individual cell and the changes which take place in it represent, in general, the organism as a whole, further study was made upon fresh, animal cells of various kinds; leucocytes, brain cells, anterior horn cells of

the spinal cord, posterior root ganglion cells, and cells from some of the body organs, including the liver, kidney and spleen.

The methods used were those of the biologist, in studying ultramicroscopically the effects upon such cells, of solutions of acids, alkalis and salts in different concentrations; that is, solutions which embody variations in water and salt concentrations, leading to fluctuations of electric charge. In addition, the methods of the colloid chemist were applied in the study of the blood, also with the aid of the ultramicroscope, which reveals its colloidal structure. Blood, like all dilute colloidal dispersions, is made up of a fluid dispersion medium in which small particles are seen in active motion (Brownian movement). Blood corpuscles, platelets and fibrin are, of course, also present.

It has been established that the organism strives to maintain a constant equilibrium in the cells and the surrounding fluid in a variety of physicochemical relationships. "The general fact that a certain combination of salts, usually of sodium, calcium, potassium and magnesium is required in the external medium for most forms of normal protoplasmic action, indicates the fundamental importance of the influence of salts on the structural colloids of protoplasm." [4] That sodium is present in the surrounding fluid, and potassium within the cell, and that there is normally very little interchange in these two salts has been quite definitely determined.[*]

The cell is surrounded by a so-called plasma-membrane which is considered merely as a layer of specially adapted cell protoplasm, and not as a capsular membrane in the earlier sense. This boundary surface or interface is the seat of many important physico-chemical activities. It is shown to be semipermeable, which means that it allows certain substances to pass while it holds back others. Those which pass through are, chiefly, water and substances which are lipoid soluble. Since it has been determined by analysis that the chemical composition of the cell interior is entirely different from its milieu, this membrane provides a means of maintaining an equilibrium, as long as its normal state is unaltered. This state of equilibrium is maintained in part also by the excretory functions of the lungs, kidneys, etc., the so-called "inner regulation". "The purpose of isotonicity and isoionicity is to protect the cells of the body from injury, as far as possible, by giving them a practically uniform optimum environment." [5]

It was precisely certain changes in this equilibrium between cells and surrounding medium, that we undertook to study. Various cells, as enumerated above, were examined in fresh cover-glass preparations, by means of the ultramicroscope, with the addition of solutions of salts, acids and alkalis in different concentrations.

With the dark field illumination, the colloidal structure of all cells is clearly seen. There is a general similarity, varied only by the size and number of the colloid granules. The nucleus is sharply differentiated from the cytoplasm, from which it differs in physical and chemical properties.

The swelling and shrinking of cells was the point of chief interest. According to Schade,[*] the physiological swelling balance is determined by three factors: (1) an "equilibrium" (representing a normal) of the ions contained in the surrounding fluid (i.e., 3 faint alkalinity of pH—7.45 and sodium, potas-

[4] Lillie, "Protoplasmic Action and Nervous Action," p. 170.
[*] There is probably a kinetic equilibrium, which means that there is some K outside and some Na within the cell. *J. A.*
[5] Schade, "Colloid Chemistry and Internal Medicine," in Alexander's "Colloid Chemistry," Vol. II (this volume).
[*] See paper by H. Schade in this volume. *J. A.*

sium and calcium in molar ratio of about 100.2.2); (2) the presence of a normal colloid-osmotic protein pressure in the serum; and (3) the co-operation of a normal squeezing pressure due to the mechanical resiliency of the tissue itself.

It is with the presence of normal colloid-osmotic protein pressure in the serum that we are chiefly concerned. Isotonic (isoionic) solutions produce little change in the appearance of any of the cells studied. In concentrations varying on either side of the point of isotonicity, notable alterations were at once evident, and appeared in more dilute solutions as immediate swelling of the cell due to absorption of water, the degree of swelling depending upon the degree of dilution from the optimum concentration. In the cytoplasm, the colloidal granules show some swelling individually, and enter into very active Brownian movement. This phenomenon is very apparent; the greater the dilution of the cytoplasm, the more extreme the Brownian movement. In leucocytes the swelling reached such an extreme degree in weaker dilutions, that the cell boundary-membrane burst with an explosive discharge of the contained colloid granules. In fixed cells, on the contrary, while swelling was evident and, with certain salts, especially involved the nucleus, with the addition of the presence of Brownian movement of the colloid granules, the reaction was never so extreme. This is probably due to the fact that their structure is nearer a gel form than a sol, which the leucocyte represents.

We found that higher concentrations, on the other hand (hypertonic), produced just the opposite action, causing cell shrinkage, with more or less prompt cessation of Brownian movement (where present), with increased luminosity of the colloid granules, and a final gelation of the entire cell.

These processes are both reversible up to a certain point. That is, if either concentration is brought back to, or as near as possible to, the isotonic point, by the addition of the appropriate reagent—the addition of more dilute, or water, to the concentrated, and of greater concentration to the more dilute— the cells resume a nearly normal appearance. In leucocytes this reversibility persists as long as the change is made quickly enough to bring about results before Brownian movement stops, or before the cell bursts. This reversibility is especially notable in the case of the opposed action of acid and alkali. In the brain tissue experiments, this was particularly striking. The addition of a fairly concentrated solution of sodium hydroxide (as low as 0.5%), the tissue becomes an amorphous gelatinous mass, which is promptly restored to more or less clear morphological outlines by the addition of an acid of suitable dilution. The matrix resumes its former finely granular appearance, and the cell outlines are visible again.

The reagents used in making this study were hydrochloric, nitric, sulfuric and acetic acids; sodium and potassium hydroxide, sodium, potassium, calcium and magnesium chloride, magnesium sulfate, dextrose, etc. It is not intended to imply that all of the electrolyte solutions acted in identical manner on all cells, but the changes were, in general, similar.

The somewhat specific action of calcium, and in a less degree of magnesium, upon protoplasm is generally recognized. Calcium particularly has a *toughening effect,* which was very apparent in all these experiments. This appeared as a thickening of the cell boundary, and restricted ability to swell. The fact that the nucleus failed to swell, but became rather shrunken and brightly luminous suggests a change in the permeability of the cell membrane. That there was swelling, or the tendency to swell, was indicated by the fact

that in dilute solutions the cell membrane was burst open, but only with a very small opening, and with the extrusion of but very few granules.[6] (See Joel, "Klinische Kolloidchemie," p. 71.) Sulfuric acid, too, exerted a somewhat specific influence, seen especially clearly in nerve tissue, where the cell outlines were particularly clear and sharp under its action, suggesting a special degree of coagulation of the colloid particles of the cytoplasm.

In presenting the results of this study we do not ignore the fact that *in vitro* experiments do not reproduce the exact processes of the organism, with all its compensating factors and integrated activities which tend to maintain an equilibrium, nor even of living cells in artificial media, which we propose to report upon later. It does, however, indicate that all protoplasm has a similar colloidal structure, and that this colloidal substance reacts similarly to certain physico-chemical influences. The work of Marinesco on brain and nerve tissue, as well as similar experiments by Mott, Halliburton, and others, have the same significance.

Its clinical application is clear. It demonstrates that slight changes in the body fluids may easily bring about distinct disturbance of function, without necessarily presenting morphological changes. That this can be particularly important in the nervous system appears at once as probable, since the cells here are notable for their response to irritating stimuli on account of the high lipoid content of the tissue as a whole, thus permitting an increase of liability to permeability by circulating toxins, which may be only normal constituents in abnormal concentrations.

Familiarity with neuro-pathological morphology is only necessary to enable one to make rather definite interpretations of findings from the colloid-chemical viewpoint. The presence of degrees of cell swelling involving not only nerve cells, but also those of the reticulo-endothelial system, are not uncommon. Swollen nuclei are frequently seen in the nerve cells. The classical picture of the so-called axonal reaction of nerve cells is characterized primarily by cell swelling.

In nerve tissue, however, the factor of swelling is certainly not confined to cells alone. The colloidal structure of the nerve fibres make it probable that these partake of the same condition. The difficulty of demonstrating this morphologically is clear when one considers the fluidity of their consistence, and the fact that all fixatives and mordants are very active dehydrating agents. The difficulty of demonstrating such a condition in the fresh state is obvious also.

We feel that by these studies we have demonstrated that mental and nervous disorders parallel these and similar changes in the physico-chemical structure of the nervous system. The variety of mental symptoms can be more easily understood if one recalls that morphological examination shows that in a given brain, marked changes in cell reactions may be present in one lobe or lobule exclusively; or in one convolution or a part of it, and the neighboring tissue be relatively free from any change. This matter of focal selectivity has been recognized for some time, not only in the brain but in other organs. A similar picture may be seen in a fresh blood film, where here and there, only, one sees a crenated red blood corpuscle, as well as in stained specimens where polychromatophilia is present. That these questions remain to be explained, only gives a greater interest to follow up possible indications.

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[6] Joel, "Klinische Kolloidchemie," p. 71.

The Mechanism of the Acute Inflammatory Process

By Harold A. Abramson, M.D.,

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Introduction

Many explanations have been offered to account for the emigration of polymorphonuclear leucocytes through the capillary wall to a point of injury. The views which have been most supported and for which most evidence has been gathered have been those which supposed surface tension or "chemotaxis"[1,2] to be the dominant factor in producing this emigration. These explanations have been far from satisfying.

This process of leucocytic migration is the most universal response to sudden severe tissue injury. All injuries are not followed by this response. But when there is a relatively severe injury to a tissue, leucocytes leave their customary channels and go out into the tissues to combat the injurious agents and their products. The pathologist calls this change "acute inflammation".

While there is no question that surface tension, osmotic forces, water flow, "chemotaxis" and other alterations in the tissues may play a significant rôle in producing the picture of acute inflammation, not much quantitative work on these forces in relation to leucocyte emigration has been possible because of the complexities of the involved structures. It appeared to the author some time ago, on the basis of semi-quantitative experiments, that a possible relationship could exist between the cataphoretic velocity of white blood cells and the potential differences existing between injured and relatively normal tissues.[3] Many experiments on this and related aspects of the question have been carried out since then, primarily to determine the validity of such a conception.[4 to 11, incl] These investigations are in part summarized in this article. Practically all have been made in the laboratories of Professor H. Freundlich in Dahlem-Berlin. The author desires to express here his appreciation to Professor Freundlich for his advice and criticisms which aided in every way

possible the consummation of the experiments. Some of the data are at the writing of this article unpublished.*

The reader who is not acquainted with the purely pathological aspects of the acute inflammatory process is referred to the excellent description given by W. G. MacCallum.[12]

THE SITE OF THE INFLAMMATORY PROCESS

The various organs and tissues of the body are supported and connected by fibrous connective tissue. Through the connective tissue branch the blood vessels which continue in hundreds of thousands of capillaries. It is by passage through the thin walls of these capillaries that polymorphonuclear leucocytes reach the interfibrillar spaces of the connective tissue to migrate to the focus of irritation. Here the white cells "react" with the bacteria or other chemical agent primarily responsible for the emigration of the white cells. This response to injury may take place in any organ or tissue of the body where there is fibrous connective tissue. Even though the terms "hepatitis", "gastritis", "pancreatitis", etc., are used, it must be remembered and emphasized that the migration of the polymorphonuclear leucocyte after passing through the capillary wall is in the connective tissue framework of the involved organ.

THE VISCOSITY OF THE BLOOD AND ITS RELATION TO CATAPHORESIS

As is well known, colloidal solutions may have either viscous or plastic flow.[13] While the former follows that of ordinary solutions, the latter gives values of viscosity which are dependent upon the nature of the viscosimeter and the magnitude of the shearing stress. With small shearing stresses, such plastic fluids show no flow but only a displacement. As the shearing stress increases the apparent viscosity decreases more than proportionally. The true viscosity is thus finally approached. Hess and others have reported that serum has viscous rather than plastic flow.[14] This point is in need of reinvestigation. The viscosity of blood varies only slightly with the shearing stress, as Table 1 shows.[14] The fact that serum and blood show here almost

TABLE 1.

When red cells are present in serum in great numbers, the flow of the blood through a capillary is that of a very slightly plastic fluid. These figures are from Hess.

Pressure in mm. Hg, with which blood was forced through capillary of viscosimeter	42	38	25	11.6	7.8
Viscosity	5.8	5.8	5.8	6.08	6.2

no plasticity in flow is important in considering the nature of the interfibrillar fluids of connective tissue before and after injury. Loeb, Atchley and Palmer have shown that the make-up of transudates may be explained by ordinary laws of membrane equilibria.[15] It may therefore be expected that somewhat

* Most of the unpublished data is to be published in the *J. Gen. Phys.*, Vol. II, in press.
[12] MacCallum, "Pathology," Philadelphia, 1924, pp. 132-155.
[13] Freundlich and Schalek, *Z. physik. Chem.*, 108, 153 (1923); Bingham, in Colloid Chemistry, New York, Ed. by J. Alexander, Vol. I, p. 720; Herschel, *ibid.*, p. 727; Hatschek, *ibid.*, p. 738.
[14] Hess, *Pflüg. Arch.*, 162. 187 1915).
[15] Loeb, Atchley and Palmer, *J. Gen. Physiol.*, 4, 591 (1921).

similar processes are responsible for the composition of the interfibrillar fluids in connective tissue. With injury, certain changes take place. Serum albumin, globulin and fibrinogen flood the injured area.[16] There are also changes in the nature and quantity of the electrolytes present, particularly of hydrogen ions.[17, 18]

The preceding sketch of composition of the medium (omitting for the present the capillary wall) which constitutes the pathway for leucocyte migration, gives rise to several queries concerning the nature of the resistance offered
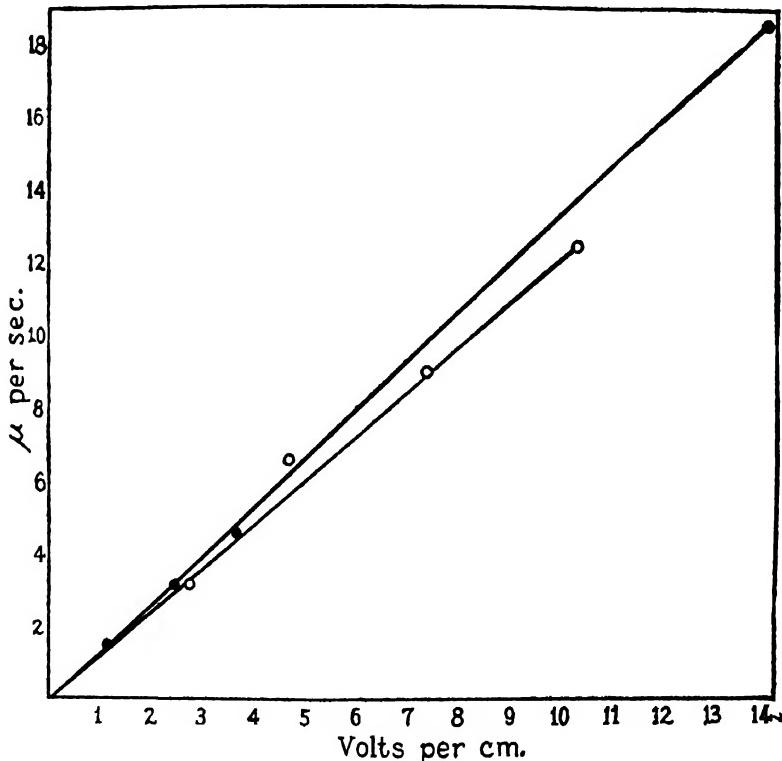
Fig. 1.—The cataphoretic velocity of red cells in plasma is proportional to the drop in potential applied. The ordinate values are relative.

by these media to leucocyte migration. It is well known that leucocytes can move by virtue of their amoeboid movements through preparations of plasma or fibrin gels. What the influence of such a fluid like plasma on migration would be if the movement of the blood cell were due to cataphoresis, will now be considered. The cataphoretic velocity of microscopic particles (red cells were more conveniently studied) in plasma was found proportional to the applied difference of potential. (Fig. 1.) The same relationship held for serum. (Fig. 1a.) A similar experiment was performed with a five-hour old isotonic solution of 0.5 per cent gelatin which had plastic flow. This also
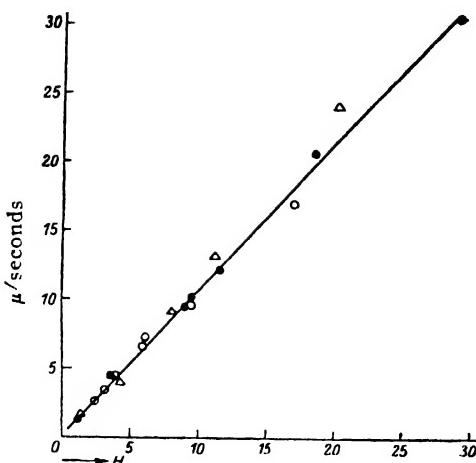
Fig. 1a. The cataphoretic velocity of red cells in serum is proportional to the potential drop (H) applied.

showed the same straight line relation. (Fig. 2.) These experiments demonstrate that for media whose flow is slightly plastic, small differences of potential produce cataphoretic migration which is independent of the plastic properties of the fluid. From this it follows that the cataphoretic velocity of microscopic particles in fluids showing only slight deviations from Poiseuille's law is expressed by the formula:

$$V = K \frac{H}{\eta} \qquad (2)$$

where V = the cataphoretic velocity, H = the difference of potential and η = the viscosity where K is a constant. That this relation is true for certain gels will be shown in detail later in this paper.

The above direct proportion between force and the movement produced does not hold for other types of forces examined which result in movement of a microscopic particle. For example, as Freundlich and Seifriz [10] have shown, a nickel particle suspended in a medium having plastic flow moves a short distance upon application of a unipolar magnetic field of slight intensity.
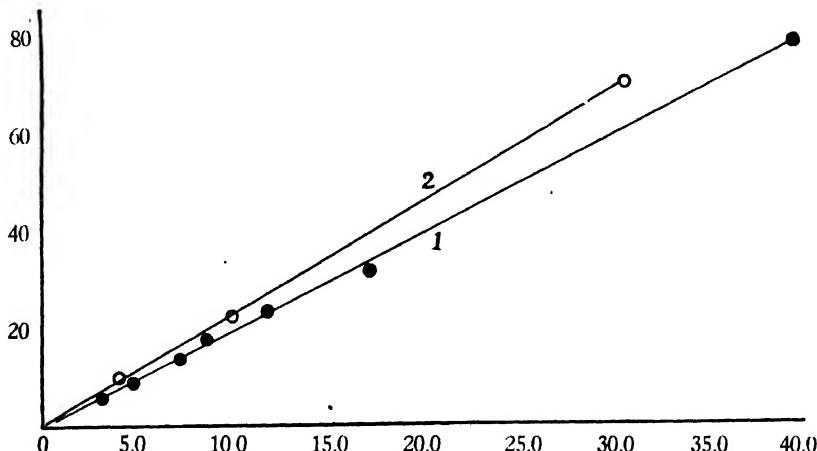
Fig. 2 —During the ageing of a 0.5% gelatin sol, the solution assumes the properties of a liquid having plastic flow. These curves show that the addition of this new structure to the sol has no influence on the cataphoretic velocity. The velocity-e.m.f. curves made at 1 hr. (Curve 1) and at 5 hrs. (Curve 2) demonstrate that the changes in apparent viscosity mentioned are without influence. The speed is therefore inversely proportional to the true viscosity and directly proportional to the e.m.f. The viscosity may be direct function of the true viscosity.

[10] Freundlich and Seifriz, Z. physik. Chem., 104, 233 (1923).

The particle then stops, and upon cessation of the current returns to its original position.

It is interesting to note that if blood exhibited plastic flow to a considerable degree, the pressures needed to force it through the capillaries would be so great that a great difference in the structure and function of our blood-vascular system would be necessary, all other things being equal.

The Cataphoresis of the Blood Cells of the Horse. The Surface of Blood Cells

Dineur in 1893 [20] was perhaps the first to sense that the electrical charge on leucocytes was in some way connected with leucocytic emigration. He placed platinum electrodes into the normal and inflamed peritoneal cavities of the frog and attempted with a very crude technic to study leucocyte cataphoresis. His results and those of Schwyzer [21] and others have been most contradictory. This is reviewed in detail elsewhere.[5]

The author used a microscopic method similar in most respects to that described by Northrop [22] to determine the absolute cataphoretic velocity of polymorphonuclear leucocytes, lymphocytes and red cells in serum and plasma. The velocity of the fluid, V_m, at different levels in the cataphoresis cell was easily measured. Then the absolute velocity, V, of a particle at a given level (for example a polymorphonuclear leucocyte or lymphocyte) is

$$V = V_o - V_m \tag{1}$$

where V_o is the observed velocity.

Plasma suspensions of red cells and white cells were obtained by oxalating 1 liter of freshly shed horse blood with 8.5 cc. of the saturated solution of the potassium salt and allowing most of the red cells to settle out. The supernatant fluid after 1-2 hrs. had a sufficient number of red and white cells to make the present studies convenient. Red cells in this medium were negatively charged and migrated to the anode with a mean speed of 0.98 μ per sec. per volt per cm. (seven horses). The values varied between 0.90 μ and 1.07 μ. Similar measurements of the red cell velocity in serums from 10 horses gave a mean value of 1.0 μ per sec. per volt per cm. These values varied between 0.90 μ to 1.08 μ. Keeping the cells on ice for periods as long as three days resulted in no significant change in cataphoretic velocity. It is evident that neither the presence of the fibrinogen nor of the oxalate ion in the plasma influences appreciably the migration of the red cells. Slight changes in pH occurring while standing seem also without appreciable effect. As the isoelectric pt. of leucocytes has not been studied, the effect of large changes in pH is not known. (See the curves given by Netter.[23]) The cataphoretic velocity of polymorphonuclear leucocytes studied in the plasma of the same group of horses for which red cell migration has just been given, varied between 0.51 μ to 0.59 μ per sec. per volt per cm. These cells were also negatively charged. The mean velocity was 0.54 μ per sec. per volt per cm. Subsequent measurements with another cataphoresis cell have given slightly lower values, but these need not concern us here. The ratio of red cell to polymorphonuclear leucocyte speed is 98/54—1.8. The presence of blood platelets *

in plasma lessens the ease with which leucocytes may be differentiated from lymphocytes because of optical difficulties. For this reason a greater number of measurements have been made with serum. As before, leucocytes and lymphocytes were studied with the red cells simultaneously. Polymorphonuclear leucocytes migrated to the anode with a mean speed of 0.51 μ per sec. per volt per cm., varying between 0.47 μ and 0.55 μ. Small lymphocytes, negatively charged, wandered with a mean speed of 0.60 μ per sec. per volt per cm. The variation here (five experiments) was between 0.54 μ and 0.66 μ. As noted before [3] for human lymphocytes, keeping the white cells of both types on ice for two days had no perceptible influence on the speed of migration. It is also significant that preliminary measurements with degenerated spherical non-amoeboid polymorphonuclear leucocytes have given similar values.

The fact that lymphocytes migrate 20 to 30 per cent faster than leucocytes is most interesting. It points to a distinct difference in the surface of these two types of white blood cells. That red cells migrate with a different velocity could have been expected, perhaps, from the fact that the lipoids present in the red cell surface introduce there a different adsorption mechanism.* An idea current in the literature is that lipoids, bacteria, inert substances, and other particles are all immediately surrounded by an adsorbed protein layer which is the same regardless of the nature of the adsorbing substance.[24] These experiments with red cells point distinctly to a state of affairs quite the contrary. The beginnings of an attempt to determine the nature of this difference were made with quartz particles in serum and gelatin-serum mixtures.

Quartz particles surround themselves under certain conditions in very dilute solutions (e.g., 10^{-4} to 10^{-5} gm. per cc.) of gelatin and albumin, with protein films. The particles then behave as if they were particles of the protein. In serum, which contains about 7 per cent of protein, the cataphoretic velocity of the quartz particles should be that produced by a film of the serum proteins. This has been mentioned also by Fenn.[25] * Table 2 shows that quartz

TABLE 2.

The cataphoretic velocities (C.V.) of quartz particles and polymorphonuclear leucocytes in sols like serum and .7 per cent gelatin serum are identical (within the limits of experimental error). The serum contained about 1/10 of .9 per cent NaCl by volume. Values are relative, measured in the middle of the cataphoresis cell. The decidedly lower values for gelatin serum are due to a lower E.M.F. in the cataphoresis cell.

	Quartz Particles	Leucocytes	Red Cells
	C.V. in μ/Sec.	C.V. in μ/Sec.	C.V. in μ/Sec.
Serum	7.9	7.5	13.5
.7 per cent gelatin serum	3.7	3.9	6.2

particles and polymorphonuclear leucocytes migrate (within the limits of experimental error) with the same cataphoretic velocity. Addition of 0.7

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* The experiments of Mudd and Mudd (*J. Exp. Med.*, **43**, 127 (1926); *Biochem. Z.*, **186**, 378 (1927) show beautifully some of the differences in cell surface composition as indicated here. These experiments should be consulted.

[24] Eggerth and Bellows, *J. Gen. Physiol.*, **4**, 679 (1922).

[25] Fenn, *J. Gen. Physiol.*, **4**, 373 (1922); *ibid.*, **5**, 149, 163 (1923), has interesting and suggestive data.

* Fenn quotes the work of Ravis.

per cent of gelatin to the serum produced no material change in cataphoretic velocity. One is tempted to conclude that the surface of leucocytes in such media is determined by precisely the same conditions which determine the surface of quartz particles. Red cells, on the other hand, seem to be affected differently by the presence of protein while the surface of the lymphocyte represents an intermediate type. The effect of "washing" on blood cell suspensions, the influence of proteins on leucocytes, red cells, and on lipoid and protein films are problems which must be solved before a more exact analysis of the foregoing data can be offered.

THE INFLUENCE OF THE SHAPE OF THE PARTICLE ON CATAPHORETIC VELOCITY. THE HELMHOLTZ-LAMB EQUATION

The relation between the cataphoretic velocity of leucocytes and their shape was not only interesting from the point of view of the migration in tissues but also because of the theory of Debye and Hückel [26] and its connection with the calculation of the electrokinetic potential. These authors have maintained that the cataphoretic velocity should be a function of the shape of the particle. They suggested that the factor $1/4\pi$ of the Helmholtz-Lamb equation [27]

$$V = \frac{1}{4\pi} \frac{HD\zeta}{\eta} \qquad (3)$$

(V = velocity, H = potential drop per cm., D = specific inductive capacity of the medium, ζ = electrokinetic potential, η = viscosity of the medium, all units C.G.S. electrostatic) was correct for cylindrical particles. The factor $1/6\pi$ was proposed to replace $1/4\pi$ when the particles were spheres.

No variation in the shape of the leucocytes could account for the slight differences in cataphoretic velocity which were found. These differences were always within the limits of error of the experiment. All conceivable amoeboid forms have been studied. Further confirmation of this fact, that the cataphoretic velocity is independent of the shape of the particle, was found in experiments with red cells, crenated cells, red cell rouleaux and clumped red cells, as for example in Figure 3. In

FIG. 3.—Single red cells and red cell aggregates in serum migrate cataphoretically with the same speed. The Greek Zeta potential was about 24 millivolts, thus above the critical potential where rapid coagulation takes place. These variously shaped particles are one of many groups studied which, within the limits of the method and in the microscopic range studied, show cataphoretic velocity to be independent of the shape of the particle.

these experiments single cells and agglutinated cells (rouleaux) migrated with the same speed. The fact that cataphoretic migration is independent of the shape of the particle was finally demonstrated for particles of quartz,

[26] Debye and Huckel, *Physik. Z.*, **25**, 49 (1924).
Huckel, *Ibid.*, **25**, 204 (1924).
[27] von Smoluchowski, in Graetz, "Handbuch der Elektrizität und des Magnetismus," 1921. Vol. II, p. 374 *et seq.*
Michaelis, "Colloid Chemistry," ed. by J. Alexander, New York, 1926, p. 471.

glass and other inert particles suspended in distilled water and in solutions of crystalloids.

It is evident that the calculation of the ζ-potential of blood cells involves certain difficulties but for the sake of future reference such calculations will be made here. It has been shown that η, the observed viscosity, may be taken as the true viscosity of the serum or plasma.* Taking 0.0185 for η and 85 for D (Fürth), and keeping the factor 4π in equation (3), the electrokinetic potential at 18° for a drop in potential of 1 volt per cm. is

$$\left(\frac{4 \times 3.14 \times .0185 \times (300)^2}{85}\right) \quad (V \text{ in } \mu \text{ per sec.}) \text{ millivolts}$$

or

$$(24.5 \times [V \text{ in } \mu/\text{sec.}]) \text{ millivolts, at room temperature.}$$

Table 3 gives the calculated values of ζ for serum. Similar calculations may be made for oxalated plasma, whose viscosity is 5 to 10 per cent higher than serum.

TABLE 3. *The Electrokinetic Potential of the Blood Cells of the Horse in Serum.*

	C.V. $\mu/\text{sec.}/\text{volt}/\text{cm.}$	ζ-Potential Millivolts	Remarks
Red cell	1.0	24 5	The means of a few similar experiments, m a d e subsequently with another cataphoresis cell, were about 10 per cent lower t h a n t h e values given here
Polymorphonuclear leucocyte ..	.51	12.5	
Small lymphocyte	.60	14.7	

It is noteworthy that single and spontaneously agglutinated red cells have a ζ-potential which is about three times as high as the critical potential (the minimum ζ-potential for relatively rapid agglutination) given by Northrop and Freund [28] and Oliver and Barnard [29] * for red cell suspensions. Freundlich suggests that there may be two critical potentials.[30] Between the first and second, slow coagulation may take place. The second critical potential, probably corresponds to that value given by the authors just cited where rapid coagulation occurs. Whether agglutination may take place, contrary to the accepted view, without any change in ζ (isopotential agglutination), is a matter for future investigation.

THE PREPARATION FOR EMIGRATION

The stickiness or adhesive property of the surface of leucocytes is a particularly important factor in preparation for emigration. It is this type of cell (in contra-distinction to the red cells and lymphocytes) which sticks to the capillary wall. In tissue injury, the polymorphonuclear leucocytes which prior to the injury had had a spherical shape in the blood stream, acquire the

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* It has been called to my attention by a colleague that it is possible that the value of η for white cells and quartz particles may require a constant correction factor under certain conditions. e.g., when the quartz particles migrate with the same speed as the protein molecules of the medium. The question is not pertinent for the argument here. But it is well to keep in mind the difficulties present.

[28] Northrop and Freund, *J. Gen. Physiol.* 6, 603 (1924)

[29] Oliver and Barnard, *J. Gen. Physiol.* 7, 99 (1915)

* Oliver and Barnard have much related data on red cell cataphoresis

[30] Freundlich, "Colloid and Capillary Chemistry," London, 1926, p. 432.

property of sticking to the capillary wall at the site of injury. Tiny pseudo-podes are seen to stick to the wall as the cells roll by. This must be primarily dependent upon changes at or near the capillary wall rather than upon changes in the leucocytes as leucocytes in capillaries other than those at the site of injury do not show this property of adhesiveness. The capillary wall, which is composed of an extremely thin and flattened out layer of endothelial cells, has a surface in contact with the blood stream. Whether this surface behaves in regard to the adsorption of proteins from the blood like the leucocyte, lymphocyte or red cell is unknown. In a suspension of horse cells in serum, cells only of the same sort clump together. That is, one sees clumping of red cells, or leucocytes, or lymphocytes, but intermingling is rare. The adhesiveness of the cells seems to depend upon a similarity of the joining surface film—just as in the case of cementing two smooth surfaces together. This is brought out beautifully by the behavior of the "glass" floor of the cataphoresis cell. In the presence of protein, this "glass" floor is covered by a thin film of adsorbed protein. This adsorption of proteins by glass is demonstrated in Figure 4. Here the ordinate values are relative speed in μ per sec. of quartz particles in dilute albumin solutions. The abscissa values correspond to initial gms. per cc. of albumin. As is evident from the curves, all the glassware used in mixing must have also adsorbed protein. The amount of albumin left for adsorption is, therefore, much less than noted. Curve I represents the speed of the water against the floor of the glass cataphoresis cell. Curve II is the cataphoretic velocity of quartz particles. It is evident that slight traces of protein, lower than 10^{-7} gm. cc., may affect the surface of quartz and glass. A similar adsorption takes place on both sides of the isoelectric point and in sufficiently concentrated solutions, e.g., 10^{-4} gm. cc., the quartz particles have the same cataphoretic velocity as the pure protein.

Let us now return to the adhesion of leucocytes to a glass surface in the presence of serum. It has been pointed out that quartz particles coat themselves with serum protein so that their cataphoretic velocity is the same as that of leucocytes. Glass particles also do the same. It seems likely, on the basis of the foregoing, that leucocytes stick to a glass surface of the cell, even in the presence of powerful streams of fluid sucked through the cell, because of similarity in surface film. The red cells and lymphocytes whose surfaces have been shown to be different from the leucocyte do not stick to the "glass". One may look for the same mechanism in the capillary wall. Incidental to injury, a change in the endothelial surface may take place, perhaps similar in some ways to the surface changes of the red cells described by Coulter.[31] The normal surface is probably like none of the cellular elements of the blood. It is well known that with injury the capillary wall becomes more permeable for proteins. This change in permeability leads to a change in surface adsorption of protein and should provide not only a new surface but a surface something like that of the leucocyte producing a surface for adherence.[*]

The cytoplasm of leucocytes is similar in many ways to a liquid having plastic flow. Perhaps the word "elasticity" used by the Germans [32] describes

[31] Coulter, *J. Gen. Physiol.*, **3**, 309 (1922).

[*] Injury of a vessel wall producing a platelet thrombus may be explained in the same way. It has been mentioned that blood platelets seem to have the same surface composition as leucocytes. When platelets are absent from the blood stream, the thrombus may consist entirely of leucocytes and fibrin. Aggregation of platelets in plasma may take place without any significant change in electrokinetic potential. Hence, all that is needed for the thrombus to form is the surface change on the capillary wall, which permits adhesion between platelets and endothelium, followed by aggregation without change in electrokinetic potential.

[32] Freundlich and Schalek, *Loc. cit.*

more easily what is meant here. Long strands of cytoplasm are drawn out by the sticky capillary wall and these then retract just as if they were a substance like rubber.* A particularly interesting instance of such a phenomenon has been observed by the author. One hour after a frog had been anesthetized by urethane, the microscopic changes produced incidental to a needle HCl
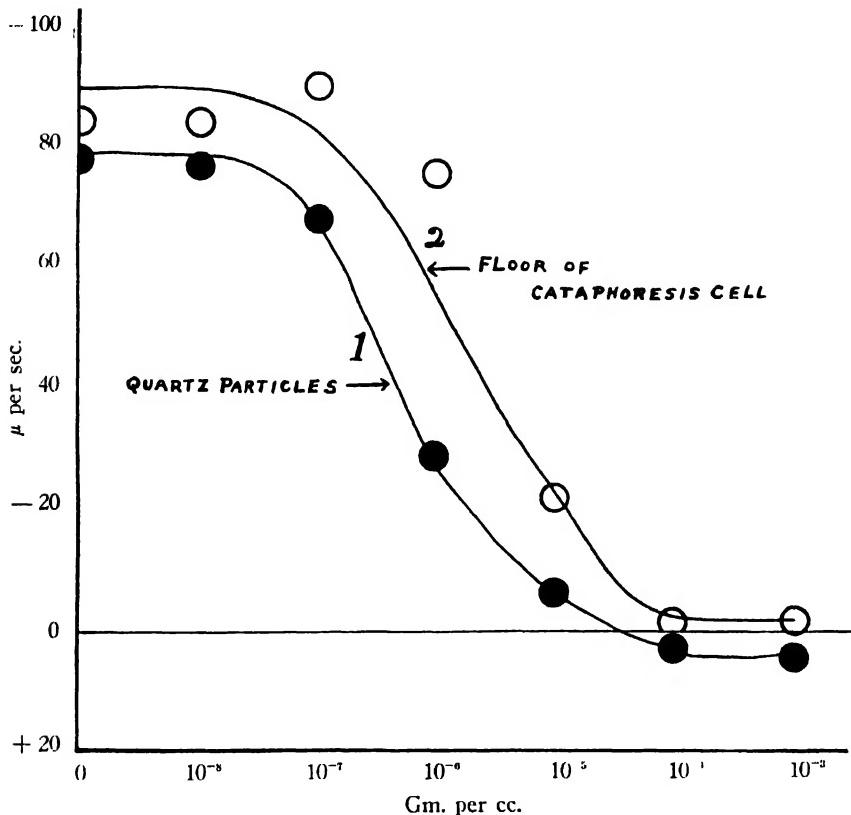
Fig. 4.— The effects of minute traces of protein on the electrokinetic potential of quartz and glass. The concentrations are *initial* values; and since the mixing cylinders absorbed protein, the values given are too high. Where the curves are parallel to the abscissa, the C.V. of the quartz particles is identical with that of the pure protein. Davis has noted that glass adsorbs serum protein Such adsorbtion takes place in *dilute* solution on both sides of the iso-electric pt.

puncture of the web, were studied. The vascular channels were wide open with a very rapid flow of cells. At the bend of a capillary, about four times the width of a white cell, an amoeboid cell was seen to be suspended in a rushing blood stream by a single thin white thread of cytoplasm about three times as long as the cell itself. (See Fig. 5a.) This cell, although thus attached to the capillary wall, was in the center of the capillary and of the blood stream, surrounded and tossed about by red cells streaming past so rapidly that it was the only cell to be made out clearly. The diameter of the pseudopod

* See paper by Robt. Chambers in this volume. *J. A.*

where it was fixed to the wall was perhaps about 1 μ. The thin pseudopod then suddenly shortened and the cell approached to within its own length of the capillary wall. (Fig. 5b.) Then, overwhelmed by the force of the blood stream on the cell itself, the pseudopod was again stretched out to its previous length. (Fig. 5c.) Such play in the center of the capillary went on for several minutes when the cell was torn away from its pseudopod mooring. Figs. 5d and 5e.)

It is evident that the adhesive force of this pseudopod was at least equal to the resultant forces of the blood stream and of gravity on the white cell.

———→ indicates direction of blood flow.
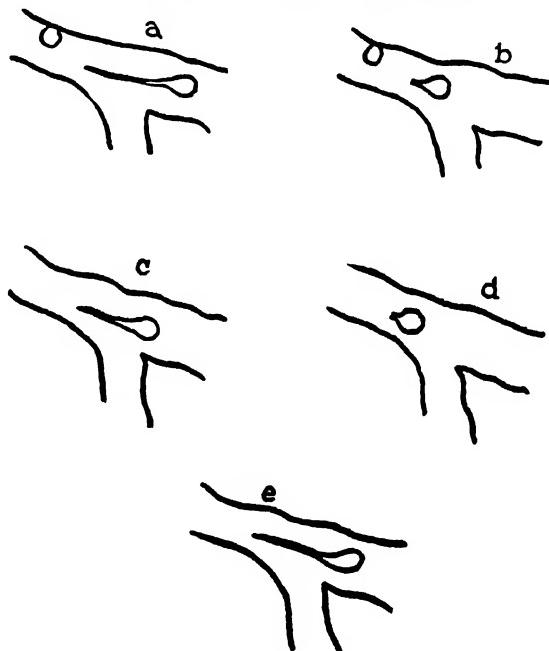
Fig. 5.—See text for description.

As the preparation was in the horizontal plane, we may neglect the force of gravity. The frictional resistance, F, of the cell in the stream is then equal to the adhesive force of the pseudopod. To obtain an idea of the order of magnitude of this force, let it be assumed that the leucocyte of radius, r, was in a wide tube with a fluid of viscosity, η, and having a velocity, V, usual for that of blood in the capillaries. Then

$$F = 6\pi r\eta V \qquad (4)$$

Substituting 4×10^{-4} for r, 0.03 for η and 0.05 for V, the value of 10^{-5} dynes is obtained for F, the adhesive force of the pseudopod. It has been mentioned that the cell was maintained by the blood in the *center* of the stream. The force of the blood stream at the center tending to tear a leucocyte away from the capillary wall is much greater than along the wall. A leucocyte

usually touches the wall with many pseudopods. And since the adhesive force which may be exerted by one pseudopod is probably more than enough under the conditions of the above observations, leucocyte arrest is more easily understood. Fenn[33] has made most interesting *in vitro* studies of the adhesiveness of leucocytes to glass, mica and other surfaces. Until more is known of the influence of traces of proteins on such surfaces, much difficulty may be encountered in the interpretation of Fenn's results.

Fenn and others have contributed the theory and experimental work related to the spreading of leucocytes on solid surfaces.[34] Harkins[35] has recently discussed the spreading of liquids upon solid surfaces. "A liquid will spread if its work of surface cohesion, W_c, is less, and will not spread if its work of surface cohesion is greater than its work of adhesion, W_a, with respect to the surface of the liquid or solid upon which the spreading is to occur. The *spreading coefficient* which under the conditions hereafter specified gives a measure of the tendency to spread, is defined as

$$S = W_a - W_c. \tag{5}$$

Considering the spreading of a leucocyte on the capillary wall, when the work of surface adhesion, W_a, is greater than the work of surface cohesion plus another factor, R, which we may designate as the rigidity of the leucocyte, S, is positive and spreading preparatory to emigration occurs when

$$W_a > W_c + R \tag{6}$$

The Potential Differences in Injured Connective Tissue and the ζ-Potential of Leucocytes

The leucocyte has been left spread out on the capillary wall preparing for emigration. In rigid chronological sequence the migration through the gel-like capillary wall should be taken up at this point. The discussion of the mechanism of this part of the process is omitted here, however, until the last part of the paper. Let us assume for the present that the leucocyte has wandered through the capillary wall, and take up the mechanism of migration to the injured area from this point.

It has been stated that the studies here presented were made in an attempt to correlate the cataphoretic velocity of white cells with the potential differences passing between injured and relatively uninjured tissues. Other views such as those which lay emphasis on surface tension, "chemotaxis", etc., are not meant to be excluded. The author feels rather that all the physico-chemical changes which accompany tissue injury must coordinate to produce leucocytic migration. For the present, however, emphasis is laid upon the electrical changes present. As mentioned previously, the migration to the point of injury takes place through the interfibrillar spaces in media whose properties of flow probably resemble plasma or serum. Although this pathway seems fairly homogeneous microscopically, a new series of reactions take place incidental to injury. The increase in electrolytes has been studied by Schade.[36] Rous,[37] Schade[38] and others have demonstrated an increase in the concentration of hydrogen ions at the point of injury.

[33] Fenn, *J. Gen. Physiol.*, *loc. cit.*
[34] Fenn, *Ibid.*, *loc. cit.*
[35] Harkins, "Colloid Chemistry," ed. by J Alexander, New York, **1926**, p. 192 *et seq.*
[36] Schade, *Loc. cit.*
[37] Rous, *Loc. cit.*
[38] Schade, *Loc. cit.*

Gessler [39] has similarly found an increase in oxidative processes. All these processes produce a new physico-chemical system at the injured focus which are connected with the blood stream by the fluids which constitute the pathway of leucocytic migration. This new chemical system produced by injury sets up not only these differences in surface tension, in viscosity, in osmotic pressure, etc., but in consequence of these also differences in potential which

Schema to show path of electrons in injured tissue
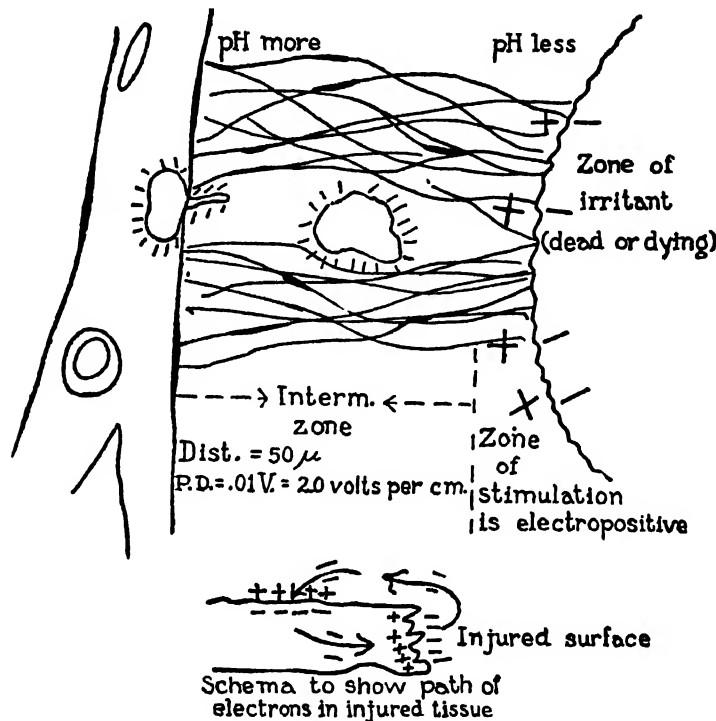
FIG. 6.—This diagram shows schematically the explanation advanced to account for the migration of a leucocyte to a point of injury in the interfibrillar spaces of connective tissue. (A later figure is given for the emigration through the capillary wall.) See text and Table 4 for further details.

always accompany such changes. The study of artificial cross-sections of other living tissues has made the difficult problem of determining the nature of potential differences passing between capillary wall and point of injury simpler.

It is well known that the surfaces of injured muscle, and of tissues and plants in general are electronegative to the relatively uninjured surface.[40] That is, electrons flow from the relatively normal to the injured zone which is electropositive (Fig. 6). The same conditions probably exist in injured connective tissue following injury.

[39] Gessler, Arch. Exp. Path. Pharmacol., 92, 273 (1922).
[40] du Bois-Reymond, "Untersuchungen uber thierische Elektricitat," Berlin, 1848; Hermann, "Handbuch der Physiologie," Leipsic, 1, 192 (1879); Biedermann, "Elektrophysiologie," Jena, 1895, 274; Beutner, "Entstehung elektrischer Strome in lebenden Geweben," Stuttgart, 1920; Fujita, Biochem. Z., 118, 11 (1925).

The change in kind and concentration of ions produces a zone of excitation which should be electropositive to the blood stream, to capillary wall and to the tissues adjacent thereto. (The rôle played by the capillary wall will be discussed later.) There would be then a drop in potential between capillary and injured zone. The concentration gradient of the ions in the intrafibrillar spaces should be uniform enough to have the drop in potential fairly linear. Furthermore, it is undisputed that the concentration of hydrogen ion increases in a zone of injury. The fluids near the capillary must be well buffered.

Potential differences in tissues should be similar to diffusion potentials, except at phase boundaries. These potentials, under the conditions of tissue injury producing leucocyte emigration, should be of the order of magnitude of 1 to 10 millivolts. Where there are large differences in hydrogen ion concentration, the larger values may be expected.

Before considering these potentials in connection with leucocyte migration, it should be recalled that a drop in potential across a conductor must be expressed not only in terms of voltage, but as $\dfrac{\text{volts}}{\text{distance}}$. Thus the cataphoretic velocity (C.V.) of leucocytes has been expressed as C.V. *per volt per cm.* In a field of 20 millivolts per cm. a leucocyte suspended in plasma hardly moves. If this difference of potential were passing through a distance of 0.05 cm., the potential difference per cm. would be $\dfrac{0.02}{0.05}$ volts per cm. or 0.4 volts. Thus the differences of potential (or electromotive forces) passing between relatively normal and injured zones must be similarly converted. Table 4 gives such calculations from 0.001 to 0.100 volts for distances of 10 μ to 100 μ. These distances are of the order of magnitude usually encountered between injured area and capillary. It is evident from the table that even with very slight differences of potential, the order of magnitude of the potential difference per cm. should be sufficiently great to impose upon the negatively charged leucocyte a force sufficient to bring it to the point of injury within the usually observed time.

TABLE 4.

Estimated Potential Differences Between Injured and Uninjured Tissue	Distance Between Injured Zone and Capillary Wall			
	100μ	50μ	25μ	10μ
	Drop in Potential in Tissue			
Volts	Volts/Cm.	Volts/Cm.	Volts/Cm.	Volts/Cm.
.001	.1	.2	.4	1.0
.005	.5	1.0	2.0	5.0
.010	1.0	2.0	4.0	10.0
.050	5.0	10.0	20.0	50.0
.100	10.0	20.0	40.0	100.0

To take the lowest e.m.f. estimated (0.1 volt per cm.), a leucocyte migrating cataphoretically through a medium of the viscosity of plasma, would require only 34 minutes to reach a point of injury 100 μ distant. The potential drops in tissues are, more probably, much greater. Lower pH in the

tissues as well as higher viscosities could cause a drop in the cataphoretic velocity of the leucocytes.** The higher e.m.f.s noted in the table would, therefore, in such cases, bring the leucocyte to the point of injury well within the time mentioned above. And this time, 30 minutes to 3 hours, is that usually taken for the migration of a leucocyte to a point of injury. Even if random amoeboid movement * were mainly responsible for migration, the potential differences would give the necessary directional force.*

THE STRUCTURE OF THE CAPILLARY WALL

The semi-solid gel-like structure of the capillary wall is a system mechanically more complicated than that just described for fibrous connective tissue.[47] The cytoplasm of endothelial cells making up the wall is probably protein in composition. The fact that capillaries change their diameter, that is, dilate and contract so easily, indicates that the protein of the endothelial cells (or of other parts of the capillary wall) is similar to a colloidal liquid or gel that has "elasticity" or plastic flow. Fibrils may be present in the apparently homogeneous cytoplasm. The fact that a leucocyte can pass through the wall is sufficient evidence, however, that certain parts of the wall, at least during acute inflammation, are sufficiently soft and homogeneous to permit leucocyte emigration.

It is well known that a mechanical "injury" decreases the rigidity of colloidal solutions and gels.[7, 41, 42] This process is usually a reversible gel-sol transformation. Freundlich and his coworkers particularly have studied many of the factors which influence the speed of regulation of inorganic gels after the gel structure has been temporarily destroyed by a mechanical means, such as shaking or pressing through a capillary. Noteworthy inhibiting effects have been obtained on the gelation of an iron oxide sol with amino acids.

The word "thixotropic" has been used by Freundlich to designate this gel—sol change. It was of interest in connection with this series of investigations to see if a protein gel could be made into a soft fluid merely by shaking or by other simple mechanical means. For dilute gelatin (Lichtfilter Agfa) gels (up to 2%) this was easily demonstrable. It was also shown for more concentrated gels and even markedly stiff gels under special conditions. Other organic gels show the same phenomenon. It seems likely that the random or directed amoeboid movements of a leucocyte on the capillary wall could produce a decrease in rigidity, "a thixotropic", of the proteins or other "elastic" substances which offer resistance to leucocyte emigration. This would thus cause fluidafication of the gel-like structures in the path of emigration, with subsequent healing and re-establishing of the gel, after the white cell has migrated through the wall.

THE CATAPHORETIC VELOCITY OF CERTAIN INERT PARTICLES IN GELATIN SOLS AND GELS

Particles of quartz, zinc, or silver, were suspended in a 1 per cent gelatin sol and cataphoretic measurements demonstrated that they immediately

** Incomplete and unpublished data concerning the isoelectric point of polymorphonuclear leucocytes.
* See paper by Leo Loeb in this volume. *J. A.*
* It is of interest to consider the injured part electronegative. Granting the existence of the electromotive forces mentioned, it is difficult to conceive of the migration of the cell against the forces at play.
[41] Freundlich and Rosenthal, *Z. physik. Chem.*, **37**, 129 (1925).
[42] Freundlich and Rawitzer, *Kolloid-Chem. Beihefte*, **25**, 231 (1927).

surrounded themselves with a film of the protein. This adsorption, contrary to that reported by Loeb for collodion,[43] * took place on both sides of the isoelectric point. The same was true for air bubbles. The cataphoretic velocity of such particles was studied in a 1 per cent gelatin sol and for five hours subsequently during gelation. Table 5 shows that during this time,

TABLE 5. *Influence of Medium on Cataphoresis of Zinc Particles at Room Temperature.*

Age of Sol or Gel, in Hrs.	Character of Medium	In μ/Sec. for $\dfrac{1 \text{ Volt}}{\text{Cm.}}$
0.5	Sol	0.36
1.0	Sol	0.35
2.0	Plastic sol	0.35
3.0	Plastic sol	0.34
3.5	Soft gel	0.33
5.0	Soft gel	0.36

even though a soft gel had formed in the cataphoresis cell, the cataphoretic velocity of the particles studied remained constant. The movement of the particles is really an indicator which marks the movements of the gelatin micells because the particles and gelatin micells have the same cataphoretic velocity. The cataphoretic velocity was easily and accurately studied because in the presence of a gel the particles remain suspended for days without settling out. Thus, the movements of the gelatin micells at different levels in the cell could be easily followed. It was surprising to see the same differences in speed at different levels as predicted by the theory of von Smoluchowski for viscous liquids.[44] (Fig. 7.) According to von Smoluchowski, and as shown to be true by all investigators who have studied the cataphoresis of particles in capillary tubes,

$$V = \tfrac{3}{4}V_{\frac{1}{6}} + \tfrac{1}{4}V_{\frac{1}{2}} = V_{\frac{4}{5}} = V_{\frac{1}{5}} \tag{7}$$

That is, V, the true velocity of a particle moving cataphoretically in a cell of the type described, is found at the level 0.2 or 0.8 where the superimposed movements of the water within the cell are negligible. Laing has reported a somewhat similar phenomenon for soap sols and gels.[45] *

The fact that the structural rigidity of such dilute gels did not influence the cataphoretic velocity, was confirmed by the direct proportionality found for cataphoretic velocity and the drop in potential, the linear relationship existing for voltages between 1 to 70 volts per cm. (Fig. 8.) If the rigidity of the gel itself were significant, the cataphoretic velocity for small differences in potential should have been proportionately less.

It was concluded from these cataphoretic measurements that the true viscosity of the sol and its gel should be the same. Figure 9 shows this to be the case. The measurements were made simultaneously with those of

[43] Loeb, *J. Gen. Physiol*, **6**, 105 (1924)
* Hitchcock has observed the adsorption of protein from 2% protein solutions by collodion membranes [*J. Gen. Phys.*, **8**, 61 (1925)] The reference to Loeb in the text is for collodion particles suspended in dilute protein sols.
[44] von Smoluchowski, *loc. cit.*
[45] Laing, *J. Phys. Chem.*, **28**, 673 (1924)
* Laing showed that the transport numbers of soap and oleate micells were unchanged in the sol-gel transformation. (On this transformation, see Bradford's paper in Vol. 1 of this series.)
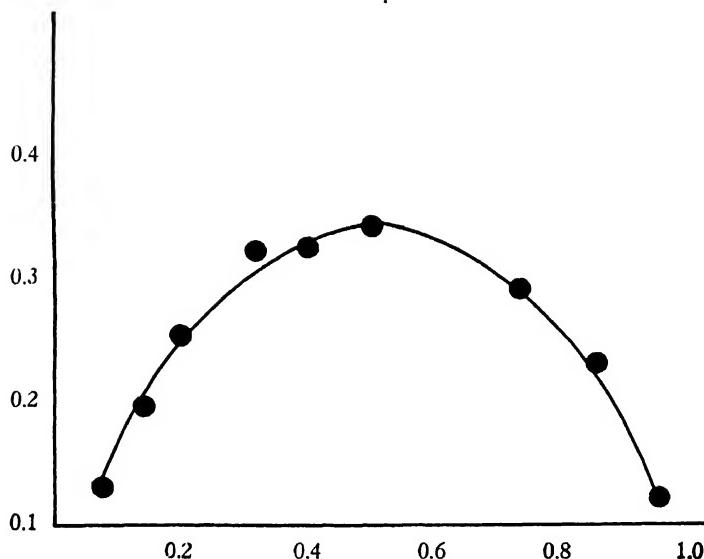
Fig. 7.—The cataphoretic migration of zinc particles in a 1.0% gelatin gel at different levels in the cell takes place as though no gel structure were present, following, therefore, von Smoluchowski's equation. The movement of fluid within the cell for this type of gel occurs as usual.
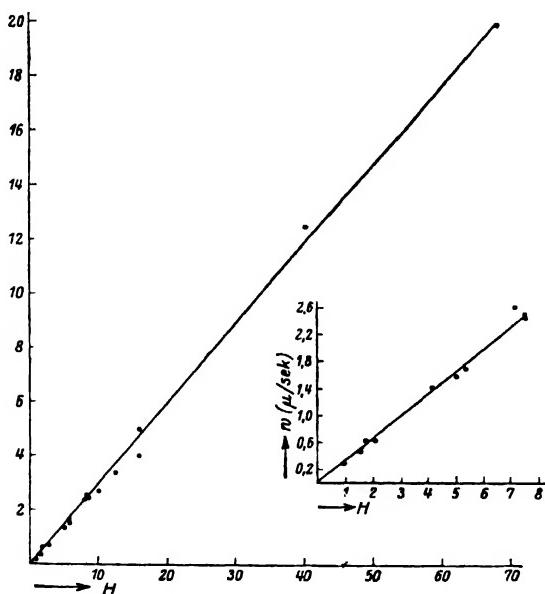
Fig. 8.—The cataphoretic migration of gelatin micells (or of inert particles coated with gelatin) in a 1% soft gelatin gel, is proportional to the applied difference of potential (H). The rigidity of the gel structure has, in such experiments, no influence on the cataphoretic velocity.

cataphoresis in a Hess viscosimeter. The ordinates in Figure 9 are apparent viscosity, η^1, referred to water. The abscissae represent the mm. Hg pressure with which the sol or gel was forced through the capillary. Where the curves tend to be parallel to the abscissa, the true viscosity, η, is approached. Deviations from the parallel represent η', the apparent viscosity, or plasticity ("elasticity") studied; there is an increase in its structural rigidity but no change in viscosity. Curve 1 has been obtained from the sol after 15 min. This curve runs practically parallel to the abscissa at the pressures used. Hence these values may be regarded as, η, the true viscosity. Curves 2 and
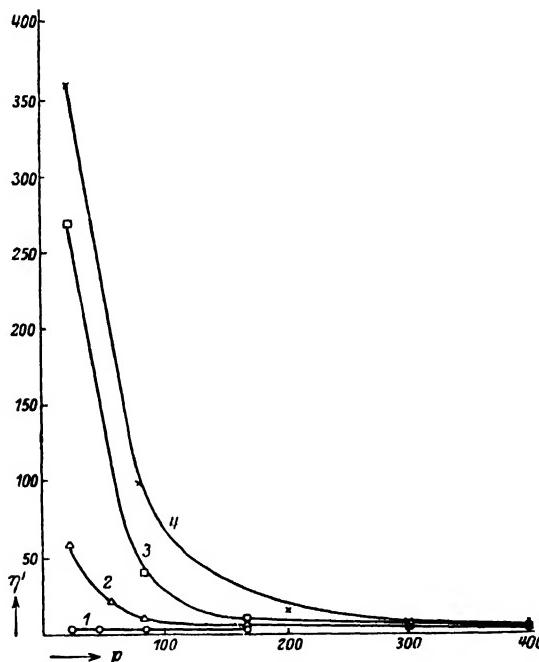
Fig. 9.—With the gelation of a 1% gelatin sol (Curves 1-4), the apparent viscosity η', rises. The values of η' at high rates of shear however, approach η, the true viscosity, asymptotically. η, therefore, remains relatively unchanged during the gelation process.

3 (75 min. and 2 hrs.) shows the well-known rigidity increase with ageing. These curves as well as Curve 4 (3½ hrs.) all approach the value of the true viscosity with sufficiently high rate of shear. Similar results have been obtained with various other plastic colloidal systems like dibenzozlcysteine and benzo-purpurin. The gelatin after 3½ hrs. had formed a soft gel. It was noteworthy that particles remained suspended in such gels indefinitely, while during cataphoresis the movement was as if a fluid of slight viscosity was present. With this type of gel (1%) there was no perceptible change in fluidity if the particles were moved to and fro over long periods.

From the foregoing the following relationships may be deduced: (1) The cataphoretic velocity, V, of certain particles in soft gels of the type described is inversely proportional to the true viscosity,

$$V = \frac{K}{\eta} \qquad (8)$$

This is the same as equation 1, but is valid for wider variations of plastic fluids. (2) For the sol-gel transformation

$$\eta' = f(F) + \eta \qquad (9)$$

where F is the shearing stress, η *remaining constant during the ageing process,** η^1 is as heretofore.

It has been shown that in serum and plasma leucocytes behave as if they were surrounded by a film of the protein of these media. In view of the foregoing, there exist, then, conditions under which leucocytes could be transported cataphoretically through a gel by virtue of an adsorbed sheath of the gel itself. The cataphoretic velocity of the leucocyte would be equal to and in the same direction as the cataphoretic velocity of the gel in which migration took place. This is further demonstrated in the following paragraphs.

THE CATAPHORESIS OF RED CELLS, LEUCOCYTES AND QUARTZ PARTICLES IN GELATIN GELS

The observations just described have not shown that cataphoretic migration may take place independent of the rigidity and movement of the gel itself. Such independent movement was demonstrated in a 1.2 per cent gelatin sol made up in horse serum, having red cells, leucocytes and quartz particles in suspension. Table 6 gives the relative speeds of these types for

TABLE 6.

The cataphoretic velocities (C.V.) of red cells, leucocytes and quartz particles in a 1.2 per cent gelatin serum gel. Relative values. Note the independent migration of the red cells.

Nature of Medium	Red Cells C.V. μ/Sec.	Leucocytes C.V. μ/Sec.	Quartz C.V. μ/Sec.	Remarks
Sol (fresh)	12	6	7	The slight decrease in velocity is due to the fact that these values are means of several measurements, w h e r e the first speeds were lower due to the presence of gel just stiff enough to reduce slightly t h e cataphoretic velocity.
After 40 minutes, soft gel	11	5	6	

the sol and the soft gel. The table shows that neither the gelatin nor the presence of a soft gel alters appreciably the relationship that has been described for serum and plasma.

In both the sol and the gel, the speed of the leucocytes and the quartz particles was about the same. *The red cells* (particularly noteworthy in the gel) *have their own distinctive velocity, about twice that of the other par-*

* That is, during the time studied. There may have been some change between zero time and 15 minutes.

be practically out of range of this difference of potential. Table 7 shows the calculated drop in potential per cm. for membrane potentials of .0001 to .010 volts. The lower values are conservative estimates. The possible potential differences per cm. calculated as above are between 2 and 1000 volts per cm. This order of magnitude of potential drop should be, if present, quite sufficient to force a leucocyte cataphoretically even through a very stiff gel.*

TABLE 7.

This table demonstrates that with even exceedingly slight membrane potentials, the order of magnitude of the potential drop per cm. should be sufficient to produce cataphoretically leucocyte emigration through the capillary wall.

Thickness of capillary wall	0.1μ	0.5μ
Possible Membrane Potentials Across Capillary Wall	Calculated Drop in Potential Across Membrane (Capillary Wall)	
Volts .0001 .001 .01	Volts/Cm. 10.0 100.0 1000.0	Volts/Cm. 2.0 20.0 200.0

Cataphoretic migration of a leucocyte could occur in two ways. It could take place as it does in the gelatin gel, i.e., moving with the proteins of the gel in the electrical field, as if it were surrounded by a sheath of the protein medium. Or migration could take place *independently* of the presence of the gel, similarly to the independent cataphoretic movement of red cells in the soft gelatin gels.* The directing influence of a difference of potential is also evident in the event that the leucocyte meet obstructions like the strands in a fibrin gel, around which it could crawl or flow with amoeboid movement. What the influence of a potential difference on this amoeboid movement would be is unknown, as all the experiments published have not excluded electroosmotic flow of the medium along the glass of the chamber containing the cells, as a complicating factor.

I should like to thank my friend, Dr. Calvin B. Bridges, for his critical help in writing this account.

* See paper by H. Schade on "Colloids and Internal Medicine," in this volume   J. A.
* It is much more likely that the leucocyte is coated with plasma protein as it passes through the capillary wall. It is well known that the proteins of the plasma flood the injured area. The leucocytes are in this flood of plasma protein. There is no reason why they should retain their plasma protein surface that has just been demonstrated. The *independent* migration has been suggested as a possible alternative, since nothing is really known of these processes as they occur *in vivo*.

Changes in Blood Concentration and Their Significance in the Systemic Treatment of Cases of Extensive Superficial Burns

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It is an axiom in physiology that the composition of the blood is constant. It is true that small variations constantly occur but within rather narrow limits blood composition under normal circumstances is invariable. When disease or other factors enter, this equilibrium of the blood may be greatly disturbed and the derangement incident to the change may lead to impaired metabolic processes or even to death.

Blood may be regarded as a complex fluid composed of colloids of various types and of a relatively large number of electrolytes. To perform its proper function of supplying nourishment to the individual cells of the tissues it must remain in such colloidal state that it is capable of passing with sufficient rapidity through the vessels containing it. Changes in viscosity, for example, may alter the character of the colloids so greatly that the heart is unable to push the thick viscid fluid through the multitudes of fine capillaries interposed in its path and so reduce the rate of circulation that the cells are subjected to partial asphyxiation with subsequent development of acidosis and eventual loss of function. An equally important function of the viscosity of the blood is in part at least the maintenance of an adequate level of blood pressure. When the viscosity is decreased appreciably the blood pressure falls, the capillaries are gorged with corpuscles and again vital processes are seriously handicapped by lack of proper blood supply. It may then be concluded that a proper degree of viscosity of the blood is of prime significance in the maintenance of normal physiological rhythm.

It may be queried—How can this desired viscosity be maintained? The most obvious ways would be by quantitative changes in the colloids (the proteins) or in the electrolytes and water or in both. In fact both methods are employed by the organism. It is quite possible for more protein to be supplied through the capillaries or for protein to be lost to the blood by both the blood and lymph capillaries, and the same statements hold true for water and the electrolytes. What determines the constancy of composition of the blood? This is one of the riddles of physiology, and although much knowledge exists with respect to how to upset the equilibrium little or next to nothing is known with respect to the mechanism of stabilizing the equilibrium.

Perhaps the most potent factor in this equilibrium is the capillary—a thin, single-celled vessel which may be regarded as a semipermeable membrane separating the blood from the lymph which bathes every functioning cell on the one hand, and on the other interposing itself between the blood and the atmos-

phere as in the case of the lungs. In either event it must be regarded as a semipermeable membrane subject in a measure at least to the laws of filtration, osmosis and diffusion, and at the same time perhaps through some other process capable of increasing or decreasing its permeability. Again the capillary has the power of dilating and contracting, thus increasing or decreasing its calibre. It is possible, although unproved, that this change in calibre may be directed by the nervous system, but it is certain that it is governed largely through hormonal action—in other words, by the state of the blood itself in ultimate analysis. Under certain circumstances the capillaries show an increased permeability for the blood colloids (the blood proteins), and again electrolytes may pass through in unusual amount. In either case water accompanies these constituents of the blood and the blood may gain or lose large quantities of water, although it is the apparent continual endeavor of the blood-regulating mechanism to maintain its content of water constant. To accomplish this it draws upon its reserves in all the other organs and tissues of the body. The greatest water stores are in the muscles and skin, although all organs and tissues undoubtedly possess this function of water storage. When for any reason water is withheld or withdrawn from the body the different tissues and organs vary greatly in their water loss. Thus, the fatty tissues, the brain, heart and bony structures lose relatively little water as compared with the muscles and skin. More than one-half of the water lost is given up by the muscles without apparent injury to either function or structure. The same statement is probably true of the skin.

Water is one of the essential requirements of the organism. In his book on "The Fitness of the Environment" Henderson has said: "In physics, in chemistry, in geology, in meteorology, and in biology nothing else threatens its preëminence. The physicist has perforce chosen it to define his standards of density, of heat capacity, and so forth, and as a means to obtain fixed points in thermometry. The chemist has often been almost exclusively concerned with reactions which take place in aqueous solution, and the unique chemical properties of water are of fundamental significance in most of the departments of his science. . . . The action of water now appears to be far the most momentous factor in geological evolution. The meteorologist perceives that the incomparable mobility of water, which depends upon its peculiar physical properties and upon its existence in vast quantities in all three states of solid, liquid, and gas, is the chief factor among the properties of matter to determine the nature of the phenomena which he studies; and the physiologist has found that water is invariably the principal constituent of active living organisms. Water is ingested in greater amounts than all other substances combined, and it is no less the chief excretion. It is the vehicle of the principal foods and excretory products, for most of these are dissolved as they enter or leave the body. Indeed, as clearer ideas of the physico-chemical organization of protoplasm have developed it has become evident that the organism itself is essentially an aqueous solution in which are spread out colloidal substances of vast complexity. As a result of these conditions there is hardly a physiological process in which water is not of fundamental importance.

"Water, of its very nature, as it occurs automatically in the process of cosmic evolution, is fit, with a fitness no less marvelous and varied than that fitness of the organism which has been won by the process of adaptation in the course of organic evolution. If doubts remain, let a search be made for

any other substance which, however slightly, can claim to rival water as the *milieu* of simple organisms, as the *milieu intérieur* of all living things, or in any other of the countless physiological functions which it performs either automatically or as a result of adaptation."

"Water constitutes more than 70 per cent of protoplasm, the structural basis of organic life. Hence it follows that water is of the greatest significance to life. Indeed by some physical chemists, protoplasm is looked upon as essentially an aqueous solution in which are spread out colloidal substances of the greatest complexity. Although we are accustomed to look upon the cell as the seat of metabolism, it is not so clearly recognized that water constitutes the medium in which the chemical changes of metabolism occur, that as a *milieu intérieur* it is essential to life, and that it is fundamental to practically all physiologic processes" (Rowntree).

Under normal circumstances the water content of the blood is constant. This constancy of water content may be regarded as one of the fundamental requirements of the organism in its endeavor to safeguard the environment of the cells. Attempts to alter the water content of the blood by introduction of even large volumes of fluid have failed to change appreciably blood composition. The water-regulating mechanism is adequate to make proper compensation quickly. It is only when this mechanism is overwhelmed either experimentally or as a result of disease that marked changes in blood composition occur. Either dilution or concentration effects may then be observed, although, in general, from studies thus far carried through, concentration is more common than dilution.

In the blood, concentration to even a moderate degree results in recognizable symptoms. The first indication noted is impairment of the circulation. When water loss becomes great the circulatory deficiency is magnified. The thick, sticky blood finds difficulty in its passage through the capillaries. It becomes an inefficient oxygen carrier, resulting in partial asphyxiation of the tissues. In consequence there may be alteration in the metabolic processes, and when the blood concentration has reached a certain level a disturbance in the heat-regulating mechanism occurs; the temperature, at first elevated falls and vital activities are suspended.

How may one gain an indication of changes in blood concentration? Of all the constituents of the blood only the red corpuscle fails to pass readily through the capillary wall. From this viewpoint it would appear that the measurement of the coloring matter, the hemoglobin, of the red corpuscle, should serve as an excellent indicator of changes in blood concentration. With certain precautions and with due consideration to possible changes in the number of red corpuscles estimation of hemoglobin content as an indicator of changes in blood concentration is unsurpassed by any other method yet proposed.

Blood concentration may be observed in a large number of abnormal conditions. Individuals exposed to high heat of the desert or to that of boiler rooms or mines often show marked blood concentration. Vomiting induced by any cause may be productive of severe blood concentration. High intestinal obstruction leads to the same result. In persistent diarrhoea much water may be lost to the organism through the stools. In Asiatic cholera and certain forms of diarrhoea in infants such loss is particularly marked and is productive of blood concentration to a degree sufficient to lead to death.

It is well recognized that in war-gas poisoning the outstanding feature of

the pathological state is the markedly concentrated blood. In certain fulminating cases of influenza a similar condition is presented and in extensive superficial burns concentrated blood may be chiefly responsible for the clinical symptoms evoked. In surgical shock and in eclampsia the blood is generally concentrated above the normal level.

Experimentally a concentrated blood may be induced by restriction of water intake, by sweating, by the action of certain drugs such as pilocarpine, by cantharides, by saline cathartics, by proteose (producing shock), by histamine, by administration of sodium chloride or urea by mouth or by the intravenous injection of sodium chloride, urea and various sugars.

From a survey of the data just cited and from the illuminating experiments of Krogh, it may be concluded that the ultimate cause of blood concentration is a change in the permeability of the capillaries. It is quite probable that under normal conditions the state of permeability of the capillaries is determined by the blood content of various hormones, such, for example. as pituitrine, from the pituitary gland, or by histamine, whose ultimate origin is histidine, or even that an interplay of other ductless glands like the adrenal and the thyroid apparatus may play a rôle. Be the exact mechanism what it may, certain it is that the capillary is of prime significance in the changed viscosity of the blood as encountered in blood concentration from whatever cause.

Thus far mention has been made of blood concentration as a menace to well being. The query naturally arises as to what constitutes a dangerous level. From a wide experience the impression has been gained that within certain well-defined limits concentrated blood, although not compatible with proper nutrition, is not necessarily a serious condition. Beyond these limits, however, life can be maintained for only a short interval. To put it differently —blood concentration up to 125 per cent of the normal value is not serious, but when 140 per cent has been reached danger enters and life is not possible for long if this limit is maintained. These relatively wide variations, which may occur without serious consequences, may be regarded as another example of the factors of safety resident in the organism.

In most of the published discussions relative to changes in blood concentration apparently little attempt has been made to differentiate these alterations and to correlate them with the types of reactions calling them forth. For example, in conditions of clinical anhydremia no distinction is made between the state existing in water starvation and that induced by extensive superficial burns, and yet there is a vast difference in the significance to be attached to the two conditions. This is especially prominent when attempts are made to restore the blood to a normal level of concentration. In the anhydremia induced by water deprivation simple administration of water rapidly restores the blood to its normal condition, whereas in anhydremia from superficial burns the restoration to normal is much more difficult. It would appear that in the two examples cited fundamental differences exist in the mechanism leading to the anhydremia. There are at least two ways in which blood concentration may be induced. In the first place one may imagine that a fluid, nearly simple water, or a dilute salt solution in composition, leaves the blood vessels in response to the proper stimulus, resulting in a more concentrated blood, a dehydration, a desiccation as it were. Or again, by a different type of mechanism, or perhaps as a result of a different form of stimulus, fluid of the nature of dilute plasma rather than of simple salt solution

passes through the vessels leaving behind a blood concentrated above the normal level. Presumably in either event there has been a change in the permeability of the capillary. In the former case this alteration of permeability may be a simple change in chemical constitution of the vascular membrane such as modification of the lipoid content or a molecular rearrangement in the protoplasm. In the other case the change in permeability must be regarded as of a more profound character, or if not different in nature must be more fundamental in degree.*

Blood concentration induced by loss of water and salts only, results in a concentration by a process of dehydration or real desiccation. This is what occurs when sufficient fluid is not introduced as by experimental water deprivation in animals, or clinically in the dehydration of infants. Again intense secretion induced by pilocarpine or by purgation with the saline cathartics will cause a rapid blood concentration. It is significant, however, that this process in general does not usually proceed to the point where a dangerous degree of blood concentration is attained. The concentration may approach the danger line but it is not maintained. This, however, is only a general statement and is particularly applicable to the saline cathartics and to pilocarpine. If actual water deprivation is pushed for a sufficiently long period blood concentration steadily mounts and death follows.

Blood concentration of this type is obviously of an entirely different nature than that induced by an inflammatory reaction, such as by extensive superficial burns. This becomes quite apparent when attempts are made to restore the blood to the normal concentration. In the former case a single administration of fluid is all that is necessary. With the concentration induced by an inflammatory reaction a single administration of fluid although helpful is not strikingly potent in permanently reducing the blood concentration. Why? Because in the latter case the capillary wall has been modified—injured—so that it is no longer capable to the normal extent of retaining introduced fluid. The new fluid runs through the injured capillaries as through a sieve. In other words, in the one type of blood concentration the capillary wall is extensively involved, in the other it is not, at least to a degree capable of measurement.

How then is it possible in blood concentration, resulting let us say, from the effects of superficial burns to produce the blood to a normal concentration? In order to accomplish this successfully fluid must be introduced into the blood *continuously*. It is true that fluid leaves the blood almost as rapidly as it is introduced, but *temporarily* it decreases the viscosity of the blood and thereby greatly facilitates its passage through the capillaries and consequently improves the general circulation, which in turn tends to maintain cellular metabolism at a level more nearly normal. This continual pouring of fluid into the blood must be maintained until the capillaries have had an opportunity of repairing their disturbed equilibrium, and this repair process takes about 48 hours. At the end of this period, under continuous fluid introduction, blood concentration gradually resumes a normal level which is maintained and the fluid then introduced is retained. As the capillaries recover their permeability the fluid intake may be reduced accordingly. The continuous fluid administration has, however, during this critical period of blood concentration tided over the organism so that vital activities have been

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* Increase in viscosity of the blood might also follow diminution of swelling capacity of the fluid blood colloids, or increase in swelling of the formed elements (erythrocytes, platelets). *J. A.*

possible. This point of continuous fluid introduction has not been sufficiently understood. To be of value water must literally be poured into the blood stream more or less continuously. Infrequent spasmodic introductions of fluid will not accomplish the object sought, namely, the transformation of a thick, viscid blood to one that the heart can readily pump through the capillaries. It does not make any different how fluid is given, whether by mouth, intravenously, subcutaneously, etc., the essential feature is to allow it to enter the blood stream continuously.

Before considering the treatment of burns it will be well perhaps to outline briefly the abnormal physiological conditions which obtain under these circumstances.

In the first place the more extensive the superficial burn the graver is the outcome to be expected, and if one-third of the total surface area of the body is affected death usually follows. Moreover, strangely enough, a deep burn is usually not so serious as one more superficial. In considering the treatment of extensive superficial burns two aspects must be given special attention. In the first place the wounded area must be treated so that it will not become infected, pain must be relieved and every attempt made to facilitate healing. These purposes are all accomplished by one type of procedure, namely, by covering the area with a thin coating of some material like paraffin which is sprayed on and forms a temporary skin over the injured area. By suitable antiseptic solutions and judicious removal of dead tissue infection may be prevented. In itself the injury to the skin is not necessarily dangerous. It is the more subtle effects -the general systemic effects, that cause death. According to one theory the absorption of the products of protein disintegration induced by the burn cause grave injury to various organs and tissues, resulting in such outspoken effects as ulcers in the stomach and intestines, damage to the liver, adrenal glands, and especially to the kidney. It is generally recognized that whatever may be the cause the systemic effects constitute the grave aspect in burned cases. The burns themselves are painful and may become dangerous because of infection, but the treatment of the injured tissue has become quite well standardized. Until recently physicians have been unable to cope with the graver systemic effects since their cause was not well understood.

Our experience with the treatment of the systemic effects of superficial burns was gained as a result of observations carried through on more than twenty victims of a theatre fire in New Haven.

The patients were admitted into the New Haven Hospital in the early evening and at once received first-aid treatment and were sent to the various wards. Blood concentration estimations were made at once. From the clinical standpoint the patients were divided into two groups: (a) those seriously burned; (b) those not so seriously burned. The blood concentration of the first group was above the danger level, namely, more than 125 per cent. Those less seriously burned were below this level. The correspondence between the severity of the clinical picture and the blood concentration was perfect. In the first group all patients were placed upon the danger list, in the second none were included in this list. The first point of significance in this investigation is that the determination of blood concentration which takes only a few minutes serves as an indicator of the gravity of the patients' condition and also points out definitely the type of treatment necessary.

In our opinion the serious condition in burn cases is the concentration of

the blood and treatment should be directed to reduce this concentrated blood to a more fluid state. The systematic treatment of these burn cases consisted simply in the forcing of fluids, water by mouth when possible, when the patient could not cooperate because of unconsciousness fluid was injected under the skin, directly into the blood, by the rectum, etc. The quantity of fluid taken in varied from 4 to 8 liters daily.

In a day or two on this treatment the blood concentration fell gradually and the patients' condition steadily improved. All patients so treated recovered, although of the group of those severely burned the vast majority could be regarded as poor risks.

From our data it would appear that water intake is responsible for the decrease in blood concentration observed in our cases, and it is quite safe to assert that without such water introduction blood concentration would not have taken the decided fall observed in every case. We believe, therefore, that the observations recorded justify the conclusion that water introduction in sufficient quantities to restore blood concentration to within normal limits is of paramount importance in the treatment of burned cases. As a result of this type of treatment, it may be stated that only two patients gave any evidences of symptoms characteristic of intoxication in burns. In these cases unconsciousness at first prevailed; this, however, disappeared after restoration of blood concentration. In all the other cases, the patients presented no untoward symptoms, such as delirium, unconsciousness, gastro-intestinal disturbance, hemoglobinuria, albuminuria, etc. Whether such facts are to be interpreted from the viewpoint that restoration of blood concentration prevented the development of conditions responsible for these symptoms, or that fluid introduction caused prompt elimination of toxic material so diluted as to be innocuous or both, remains a problem the solution of which can be determined only in the future. At any rate, from either viewpoint it would appear that fluid introduction is a rational method of treatment for extensive superficial burns.

Since the initial experience gained from the victims of the theatre fire, we have had occasion to treat a number of burned cases in the New Haven Hospital and have been consulted on cases in various parts of the United States and in general the results obtained from the treatment have been very encouraging.

How is it that the same type of mechanism is called into play in two such diverse pathological conditions as war gas poisoning and superficial burns? A little thought will show that the difference is apparent rather than real—the seat of action is the factor which makes the apparent diversity.

In a consideration of the development of pulmonary edema in gas poisoning I wrote the following: "The lethal war gases are all substances eminently irritant to living tissues and it must be accepted that the irritation produced by a gas is the initial step in the development of edema. In response to the first irritative stimulus tissue fluid finds it way to the injured area in an apparent attempt toward repair or alleviation of the injury. It is conceivable that if damage to the tissue is only slight such a procedure would result in the passage to the damaged area of only a small quantity of tissue fluid. According to this view the degree of response with respect to the local deposition of tissue fluid would be in direct ratio to the extent of injury. On the other hand, it is equally plausible to assume that this reaction may reach a breaking point at a certain degree of stimulation whereby the whole mech-

anism governing the exudation of tissue fluid is thrown out of control so that the response to the stimulation becomes overwhelming. Under these conditions a reaction which in its initial function may be regarded as beneficent, eventually becomes a direct menace to continued existence on the part of the mechanism as a whole, merely by interposing difficulties in the way of respiration and circulation." If in this quotation one substituted for war gases, heat as the irritative stimulus playing upon the skin, the mechanism is entirely similar. With burns, fluid rushes to the skin, resulting, if the skin is unbroken, in either edema of the part affected or blisters; or if the burn is more severe fluid drips from the raw surfaces. Our experience in burned cases leads one to believe that the quantity of fluid lost in this way during the first few hours after the injury may be very large, in fact sufficiently great to account for the rapid blood concentration which occurs.

In order to understand the nature of the mechanism producing the blood concentration, a word as to the character of the fluid lost from the blood is essential. From my own observations on gas poisoning it becomes apparent that this fluid partakes of the nature of plasma, diluted plasma, as it were, containing somewhat less protein than plasma, but otherwise of practically the same composition. The fact that significant quantities of proteins are present and indeed the blood proteins, particularly fibrinogen, leads to the view that the irritant factor has changed the character of the capillary wall. In ultimate analyses therefore one may conclude that the direct cause of blood concentration, in the pathological states under discussion, is due to a changed permeability of the capillary wall.

In the treatment of burns, therefore, the essential object is to keep the blood concentration near a normal level until the blood capillaries in the skin injured by the heat have had an opportunity to repair themselves and again become capable of holding within themselves the fluid of the blood in a normal manner.

Electrophoresis of Bacteria and Other Microörganisms and Some Relations to Immunological Theory

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It appears that the first unequivocal demonstration that bacteria suspended in an aqueous medium carry an electrical charge was made by H. Bechhold (1904).* He reported that this charge—as evidenced by migration in an electrical field to the anode—is *negative*. He found, among other things, that bacteria sensitized with specific agglutinin are flocculated by an electrical current. Both of these observations were confirmed by Neisser and Friedemann (1904).* Teague and Buxton (1906) * similarly found that all the bacterial species which they examined migrated to the anode, whether or no sensitized with agglutinin, but they never observed the flocculation of sensitized bacteria between the electrodes which Bechhold reported. The successive additions of salts reduced and finally abolished the migration of the bacteria and slight further additions flocculated them. In the agglutination prozone, these authors thought, the electrical charge on the bacteria is reversed in sign. Between the two electrical conditions there is an isoelectric point, a point where there is no demonstrable difference of potential between bacterium and water. In developing a theory to account for the influences of electrolytes upon bacteria they inclined to an analogy with Spiro's "Zwitterionen" and considered thus:

System	Agglutination	Migration	Structure
1. *Bact. typhosum* + agglutinin and Fe₂Cl₆ N/500.	"Vorzone"	to cathode +	H / —H \ OH⁻
2. *Bact. typhosum* + agglutinin and Fe₂Cl₆ N/5000	"Flockungszone"	none ∓	H / —H⁺ \ OH⁻
3. *Bact. typhosum* + agglutinin and Fe₂Cl₆ N/∞..	"Nachzone"	to anode ∓	H⁺ / —H⁺ \ OH⁻

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* For this and similar references see under author's name in the list of references at the end of this paper.

Buxton and Shaffer (1906) also found that bacteria migrate to the anode (i.e., are electronegative). In the same year, Cernovodeanu and Henri (1906) reported that in distilled water "Bacteridie charbonneuse, Colibacille, bacille d'Eberth, bacille de Koch, Phléole, Staphylocoque doré" are negatively charged (migrate to the anode); and that the *Bacterium dysenteriae* (Flexner) is positively charged (migrates to cathode). They found that the organisms which are negative take the basic thionin blue dye and that the positive *Bact. dysenteriae* (Flexner) does not. Conversely, the former group of organisms did not take acid fuchsin (non-phenolic) and the latter did. The electrical charges on the organisms, so far as they could observe, were unchanged by heating to 100° C. They did not succeed in reversing the sign of the charge by changing the acidity of the solution. These authors also reported that a mold which developed in tenth normal sulfuric acid showed that it carried a *positive* charge in distilled water.

Russ (1909) reported that both live and dead bacteria commonly aggregate at the anode of an electrical field, but that the direction of the migration may be modified by the addition of specific electrolytes to the bacterial suspension. Thus, he found that the tubercle bacillus proceeds to the anode in $NaCl$ solutions and to the cathode in $(NH_4)2SO_4$ solutions. In normal urine these organisms move to the anode; in a mixture of ethylamine and lactic acid the movement is to the cathode. Russ developed a simple cell to permit the collection of tubercle bacilli about the cathode. He was thereby able to find these organisms in smears prepared from the cathode solutions when, because of their sparsity, they were not to be found in smears prepared from the sediment of urine repeatedly precipitated in a centrifuge.

Thornton (1910) attempted to utilize the electrophoretic movement of bacteria and other unicellular forms as indicators for the qualitative detection of voltage gradients in fields of microscopic dimensions. He found, in general, that animal forms were electronegatively and vegetable forms electropositively charged. The latter conclusion—applying to young, active growths of protococci, pleurococcus, other algae and the following species of bacteria: "*B. typhosus, B. tuberculosis, B. diphth-avium, B. prodigiosus*, B. Lactic acid, *B. pyocyaneus, B. coli comm., B. friedländer, Sarcina lutea, Staph. aureus,* Spore-bearing bacillus, Hog-cholera and Pneumococcus"—is entirely at variance with the conclusions of later as well as earlier workers who have almost invariably found that bacteria in neutral fluids are *negatively* charged. Thornton further found that

"Bacteria from cultures which had been standing in the laboratory for some time and were not subcultured before being examined, and bacteria which, though subcultured 24 hours before use, showed very poor growth, almost immediately moved to the positive. This reversal is no doubt accompanied by marked changes in the protoplasm. In the case of an unclassified non-motile bacillus, in the laboratory of the University of Durham College of Medicine, isolated from a scarlet fever patient, a most active migration to the negative was obtained, which, tested by samples from the same agar tube kept after incubation in a cool place, lasted for about ten days, when it reversed. No further observations were possible on this organism because it soon after died out."

When examined in the same field, yeast and red blood cells were seen to move to the electrodes at about the same velocities, but the yeast to the negative and the blood cells to the positive poles.

"It may then be reasonably concluded, in so far as it is possible to have a single crucial test for so wide a range of activities, that fresh animal cells are negatively, and vegetable cells positively electrified."

This conclusion is—at least with respect to bacteria—entirely untenable except under certain rigidly described conditions which will appear later. One is led to suspect that the electrophoresis observations of Thornton were complicated by the endosmotic movement of the water in his microscopic cell and that his observations were not corrected for this possible source of error.*

In the course of an extensive investigation of the acid agglutination of bacteria, Beniasch (1911) concluded that for several species of pathogenic bacteria changes in the hydrogen-ion concentration of the suspensions did not modify their cataphoresis; that acidulation of the solutions beyond the optimum (H^+)* for the viability of the organism did not cause the reversal of the usual negative to a positive charge. Apparently, bacteria behave like protein particles or metallic sols in that their P.D. is a function of the (H^+), but they differ from many inert particles in that the reversal of the P.D.† by an (H^+) which exceeds that of the isoelectric point does not always occur. This reaction, here as for the usual sols of the colloid chemist, appears to be a function of the species of bacterium as well as of the agent used to effect a change in the P.D. It is also significant to recall that certain of the anomalous findings of Beniasch may be associated with the presence of the particular buffer salts which he used in the preparation of suspensions of specific hydrogen-ion concentrations. Schmidt (1913) found that all the species of bacteria which he studied migrated to the anode (i.e., were negatively charged). The addition of sugar to the agar culture medium, dialysis, or heating the suspensions to 60-70 or 100° C. did not affect this charge (qualitatively). The addition of small quantities of weakly acid $(N/500$ HCl) globulin suspensions caused precipitation of the bacteria. The same occurred when globulin suspensions containing CO_2 were added (K. Landsteiner had shown that immune colloids are amphoteric to anodic—i.e., negative). Schmidt found that protein-free agglutinin wanders to the anode and could not be found at the cathode; that bacteria flocculated with the usual protein-containing agglutinin preparations are amphoteric; but that bacteria flocculated with protein-free agglutinin (after being shaken up) migrate to the anode like normal, untreated bacteria.*

According to Arkwright (1914), suspensions of *Bact. typhosum* show two acid agglutination optima, one (a) at the point where $(H^+) = 3.6 \times 10^{-5}$ and another (b) where $(H^+) = 1.1 \times 10^{-4}$. The two agglutinable substances can be extracted and separated. Extract (a) is still electronegative in the agglutinable zones; often extract (b) shows no evidences of carrying a charge in solutions as acid as $(H^+) = 1.1 \times 10^{-3}$ and appears to be electro-positive in slightly more acid solutions.

According to R. Höber (1914) yeast cells are electronegative. This is contradictory to the finding of Thornton (1910) who reported that yeast is electropositive. The addition of small quantities of acid or salts of Cu, Ag, Fe or Al reverses the charge. He relates this reversible cataphoretic behaviour of yeast cells, red blood corpuscles and bacteria to the behaviour of protein and lecithin particles. Höber (1914, a) declares that *Trypanosoma equiperdum* and *brucei* are electronegative despite Traube's contrary contention that

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* Thornton's conclusion that red blood cells migrate to the positive electrode is in accord with the recent work of Coulter (1922) and others so far as neutral suspension fluids are concerned. Lillie's (1903) observations to the contrary are probably due to endosmotic interference.
* We use the symbols (H^+) and pH in the usual manner, (H^+) implying the "hydrogen-ion concentration" and pH the numerical equivalent of $\log_{10} 1/(H^+)$.
† P.D. = Potential difference.
* The paper of Walpole (1914) may be consulted for an interesting analysis of the phenomena associated with the aggregation of particles

trypanosomes carry a positive charge. In a publication of the following year Traube (1915) reaffirms that most spirochaete species are positively charged. He quotes the experiments of Commandon who found that *Plasmodium malariae, Spirochaeta gallinarum* and *Spirochaeta recurrens* wander to the cathode in an electrical field (i.e., are *positively* charged) whereas *Spirochaeta pallida* wanders to the anode (is *negatively* charged) or is stationary. The author suggests the caution with which these findings must be taken because of the streaming of the liquid under the influence of the current used to cause the electrophoretic migration of the organisms. It is important to note, in this connection, that the most recent contributions to this field of biological electrokinetics are largely concerned with technical improvements especially designed to avoid this source of error in studies on bacteria. It will be very interesting and important to repeat the work of Cernovodeanu and Henri, Thornton and others with this methodological caution in mind.[*] The significance of Traube's work in the light of chemotherapeutic mechanics is direct because of the indications from the experiments of a parallelism between the electrical charge on a particular species of spirochaete and its susceptibility (or immunity) to a specific *in vivo* therapeutic agent. It is questionable whether any extensive theoretical generalizations based upon these earlier experiments alone are justifiable until they have been confirmed.

Salus (1917), studying the electrokinetic reactions of bacteria by their adsorption on "Bolus," concluded that *Bact. typhosum* and *Bact. coli* behave like amphoteric colloids, their electrical charge changing with the acidity and alkalinity of their solution. As this author clearly recognized, his experiments did not imply that the sign of the charge on the bacteria necessarily changed qualitatively with (H'), but merely that it changed quantitatively to exceed or to fail to exceed the charge on the adsorbing agent, although qualitative changes as well may have occurred. The adsorption method supplies a relatively simple, rough technique for measuring the nature of the charge on bacteria and its approximate size.

In the brief report of the work of Girard and Audubert (1918) we meet one of the few contributions to the relations between the electrokinetics of bacteria and their biological behaviour. Using the method of cataphoresis and Perrin's form of the equation which relates the velocity of migration to potential across the Helmholtz electrical double layer,[*] they found that for the pneumococcus and for the organism of anthrax $\sigma d = 1.22$ to 0.45 (10^{-6}) C.G.S. units is associated with complete agglutination. These values are approximately equivalent to potentials of 0.017 to 0.006 volt, or 17 to 6 millivolts. We shall later have occasion to see that these values are of the same order of magnitude as the critical potentials obtained by other investigators. Girard and Audubert found that the agglutination is reversible as may be

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[*] From experiments performed recently by the writer with Professor W. H. Taliaferro it appears that *Trypanasoma lewisi* is electronegative to water, salt solution or serum by a potential difference of approximately 6 millivolts.

[*] Perrin's form is $\sigma d = \dfrac{vk}{H}$ where:

$v =$ velocity; $K =$ coefficient of viscosity; $d =$ the dimension of the double layer; σ the density of the electric layer on the bacteria; and H the electrical field strength. I, Lamb's measure of "slip," K, the dielectric constant and the constant 4π which appear in the Helmholtz-Lamb equation are omitted. If we assume that $\sigma d = V\dfrac{1}{d}$, and if we take $\dfrac{4\pi \cdot 9(10^4)}{K} = 14.(10^5)$ we may tentatively convert values of σd in C.G.S. units to $V\dfrac{1}{d}$ in volts by multiplication of values σd in C.G.S. by the factor $14(10^5)$. This procedure is only tentative and approximate. It assumes, for example, that η and K are invariables—an assumption that Winslow, Falk and Caulfield (1923) have indicated is unwarranted but for the present unavoidable.

demonstrated by shaking the clumped bacteria in solutions containing tri- or tetra-valent anions (citrate, ferrocyanide). A change of δd from 3.68 to 2.47 (10^{-6}) C.G.S. (equivalent to a change of $V\frac{1}{d}$ from 51.5 to 34.6 millivolts) is associated with a fivefold increase in the vegetative forms of *Bacillus anthracis*. For the organism of Preiz-Nocard, a change of from 3.60 to 2.38 (10^{-6}) C.G.S. (50.4 to 33.3 millivolts) was associated with a sixfold increase. A further addition of $La(NO_3)_3$ causes agglutination and a still further addition caused the development of a spore-like condition which was associated with an increased viability. A variation equivalent to the first change in δd of the Preiz-Nocard organism quadruples the *vibrion septique* (*Clostridium edematis* †). Inasmuch as these reductions in the negative potential difference between this anaerobic organism and suspension fluid were produced by additions of a salt giving the trivalent lanthanum cation the stimulation of reproduction is considered by the authors to cast doubt upon the suggested usefulness of La^{+++} ions as catalysts in oxidation processes. Changes in δd through the influence of La^{+++} ions did not appear to be associated with appreciable changes in longevity or virulence of pneumococci. A concentration of La^{+++} ions sufficient to reduce δd approximately to zero (i.e., render the organisms isoelectric with their menstruum) and make the surface tension of the bacteria maximal killed the cells but appeared to "fix" the protoplasm and preserve toxicity. For a subcutaneous inoculation of these organisms (in $\frac{1}{5000}$ lanthanum nitrate solution) gave the reaction typical of living cells. This reaction was not obtained when the pneumococci had been killed with alcohol-ether. The biological as well as the electrical effects of La^{+++} ions in the menstruum are not evidenced if a slight excess of trivalent negative ions is added. The authors therefore consider that the effects of La^{+++} ions upon growth rate, viability, virulence, etc., are caused by the electrical discharging or neutralizing properties of these ions.

Similarly, Shearer (1919) found that lanthanum nitrate, in concentrations too small to appreciably change the electrical conductivity of emulsions of *Bact. coli,* markedly affects the electrophoretic velocity of this organism. A final concentration of this salt in the medium equivalent to 1 : 50,000 halves the migration velocity; and a concentration of 1 : 25,000 nearly or completely abolishes the migration and causes the emulsion to flocculate and precipitate. In a later paper Shearer (1922) gives a more detailed report of his experiments. When using a rather crude cataphoresis cell under the dark-field microscope, he found that *Bact. coli* in distilled water, NaCl and Ringer's solution migrates to the anode. Slight traces of a lanthanum or cerium salt reduce the velocity and stronger concentrations reverse it. He also reported the rather surprising result that sodium citrate reduced and *sometimes* reversed the direction of migration. In 0.01 M. HCl, *Bact. coli* went to the anode; in 0.1 to 0.2 M. HCl, it went to the cathode. Teague and Buxton (1906) had observed a change of direction with sensitized *Pseudomonas pyocyanea* in 0.01 M. HCl. Shearer came to the conclusion that :

"The ultramicroscopic method has several serious drawbacks when applied to the study of the rate of migration of living bacteria in an electric field. In the first place the gas products formed at the poles rapidly interfere with the experiment, and secondly

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† According to the nomenclature recommended in the Final Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types, in the *Journal of Bacteriology*, **5**, 191-229 (1920).

heating effects quickly render the movements of the bacteria very irregular, sometimes entirely reversing the movement."

As we shall have occasion to indicate, these objections to the microscopical technique for measuring cataphoretic P.D. can be overcome. Working with Hardy's macroscopic U-tube method, Shearer found that *Bact. coli* in spleen broth (under layers of Ringer's solution) migrates to the anode at a velocity of approximately 1.23×10^{-4} cm. per second for a potential gradient of one volt per cm. The addition of $La(NO_3)_3$ to give a concentration of 0.00005 M. approximately halves the migration velocity—i.e., 0.86×10^{-4} cm. per sec. per volt per cm. The viscosity of his broth (with as well as without the La salt) was 11.69×10^{-3}. With a lanthanum concentration twice as high (0.00010 M.) the migration velocity was approximately zero. At this point flocculation of the bacteria occurred. Using Perrin's formula to evaluate the strength of the electrical layer on the bacteria these velocities are equivalent to $\sigma d = 1.35 \times 10^{-6}$ C.G.S. units for broth, and 1.02×10^{-6} for broth containing La or Ce salt. These values of σd are equivalent to potentials of 20.3 and 14.3 millivolts respectively—values of approximately the same magnitude reported by Girard and Audubert (1918). Shearer's broth was slightly alkaline—pH = 7.5; and his Ringer's solution had the same conductivity as his broth. He found that additions of sodium citrate up to 5 c.c. of a 20 per cent solution to 10 c.c. of bacterial suspension were without appreciable effect on the velocity of migration—a result which it is less difficult to accept than his finding with the crude microscopic cell that Na citrate reduces and sometimes reverses the charge on *Bact. coli.* Shearer confirms the finding of Girard and Audubert (1918) that a reduction in P.D. is associated with a multiplication of the organisms. Thus $La(NO_3)_3$ in spleen broth (pH = 7.5) in a concentration of 0.00005 M. lowers the σd from 1.49 to 1.03 (10^{-6}) C.G.S. (from 20.9 to 14.4 millivolts) and was accompanied by an 800 per cent growth of the bacteria. At the end of the growth period, a second addition of lanthanum nitrate sometimes did and sometimes did not effect a further stimulation to reproduction. The behaviour of Ce and Yb salts is analogous to that of the La salt. Na citrate (1-2 c.c. of a 20 per cent solution) antagonizes the effect of 0.5 c.c. of $La(NO_3)_3$ solution. The addition to a culture of salts which give negative trivalent ions was without effect on the movement of the bacteria in an electrical field; the negative charge on the bacteria was not increased. Shearer also tested the effect of lanthanum nitrate on the virulence of the pneumococcus and *B. anthracis* for mice. In a concentration of 0.0005 M. the salt appeared to abolish the virulence of the organisms, but the author considered the results inconclusive.

The publications of Szent-Gyorgyi and of Putter contribute some interesting qualitative observations on the behavior of bacteria exposed to an electrical potential. Szent-Gyorgyi (1921) prepared suspensions of bacteria in isotonic sugar solution, in Ringer's solution and in a 3:1 mixture of these two, each in a diluted phosphate buffer solution, pH = 7.0. Using a very simple microscopic cell, he found that the following organisms * were electronegative (i.e., migrated to the anode) :

> *Bacterium coli*
> *Bacterium typhosum*
> *Bacterium paratyphosum A*
> *Bact. paratyphosum B*

* Using the nomenclature of the Committee of the Society of American Bacteriologists, *loc. cit.*

Bact. dysenteriæ (Flexner)
Bact. dysenteriæ (Shiga)
Bact. typhi-murium
Bact. suipestifer
Erysipelothrix erysipelatos-suis
Pasteurella choleræ-gallinarum
Mycobacterium tuberculosis (hominis)
Mycobacterium tuberculosis (ranarum)
Corynebacterium diphtheriæ
Bacillus anthracis (vegetative and spore forms)
Vibrio choleræ-asiaticæ
Bact. melitensis
Staphylococcus pyogenes aureus
Staph. pyogenes citreus

Similarly, yeasts migrated to the anode.
Of the spirochaetes:

Sp. recurrens (African—from a mouse) migrated to the cathode (electro-*positive*)
Sp. pallida (human) migrated to the anode (electro-*negative*)

thus confirming the observations of Commandon on spirochaetes quoted by Traube (1915). Experiments with protozoa gave the following results:

Organism:	Migration to:
Paramecium caudatum	anode
Coccid from a mouse................................. .	anode
Lambia	anode
Kreuzschnabeltrypanosom (aus der Kultur).............. .	anode
Halteridium syrnii	
Flagellatenkultur aus Waldkanzblut.	anode
Flagellatenkultur aus Eulenblut (Stamm Gozony)	anode
Tryp. Theileri (Kultur)	anode
Leishmania donovani (Kultur)...........................	anode
Hamstertrypanosom (Kultur und Blut)....................	anode
Schizotrypanum Cruzi (Kultur und Blut).................	anode
Trypanosoma brucei (Blut)...............................	cathode
" *equiperdum* (Blut)	cathode
" *equinum*	cathode
" Gambiense	cathode
Stamm S.	cathode
" F.	cathode
" Braun	cathode
" Wolf	cathode
" Togo	cathode
Trypanosoma rhodiense (Blut)	cathode

Differences in the direction of migration could not be attributed to the presence of diluted blood. Szent-Györgyi suspended both anodic and cathodic species of protozoa in blood and serum after washing and centrifuging them out. The presence of added blood did not change their reactions. The relation of these findings to the specific therapeutic action of drugs, i.e., whether the effective fraction of the drug bears a positive or negative charge, is concerned with important developments in pharmácology which we cannot discuss further.[*]

Putter (1921), using the microscopic electrophoresis method of Michaelis, studied the influences of various substances on several species of bacteria.

[*] See Traube (1912); Hober (1914,a); Traube (1915); Szent-Györgyi (1921); etc.

He confirms previous investigators in this field in the finding that glass and bacteria are electro-negative to pure water and that they are similarly negative in alkaline and in weakly acid solutions. The negative charge decreases with increasing hydrogen-ion concentration. Peptone augments the influence of electrolytes in reducing the negative charge on bacteria and appears to cause a reversal of the charge when used in combination with them. Of the electrolyte ions, trivalent cations alone invert the charge without the aid of peptone, although the more common effect evidenced in Putter's data is an annihilation rather than a reversal of the charge by a salt with a trivalent cation. His finding that acid tends to discharge rather than to reverse the charge of *Bact. coli* is analogous to the findings of Loeb and of Gyemant on collodion coated with protein. *Bact. typhosum,* staphylococci and *Bact. proteus X19* (Weil-Felix) behaved like *Bact. coli.* Putter inclines to the view that bacteria evidence a special avidity for basic dyes because of these the chromogenic fraction is electro-positive, and the bacteria themselves are electro-negative.* Putter reports no absolute measurements of charge.

The publications of Northrop and DeKruif constitute the most careful and extensive contributions to the study of the electrophoresis of bacteria. Their experiments were designed primarily to cast some light on the factors which operate to affect the stability of bacterial suspensions. Northrop (1922) described an exceedingly valuable cell for microscopic cataphoresis experiments. The apparatus is designed to facilitate rapid and precise measurements of velocity of migration of bacteria in a field of determinable strength; corrections for the electroendosmotic streaming of the water are relatively simple; and the chemotactic effects of electrolysis and polarization changes are minimized by the position and design of the non-polarizable electrodes. The apparatus which has been utilized in our experiments is merely an improved form of the Northrop design. Northrop and Cullen (1922) described an inverted U-tube apparatus for macroscopic cataphoresis experiments. Northrop occasionally observed with his microscopic technique an apparent reversal of the direction of migration of the water under the same conditions that caused a reversal of the motion of bacteria. He observed that under those conditions the bacteria have become adherent to the cell walls. "The cell wall is, therefore, no longer glass but is partially composed of the same material as the suspension and therefore reverses its charge under the same conditions as does the suspension" (J.H.N., p. 633). This anomalous observation must be kept in mind when working with this apparatus. Northrop and DeKruif (1922) used a glycine, sodium phosphate, sodium acetate buffer solution and added suitable quantities of NaOH and HCl to give specific hydrogen-ion concentrations. With *Bact. typhosum* (no pH controls) they found that sodium and magnesium chlorides and sulfates reduce the P.D. with increasing salt concentration to a zero P.D. but do not cause a reversal of the charge. Cu acetate, $LaCl_3$, $AlCl_3$ and $ThCl_4$ (in this order of increasing effectiveness) reduce and reverse the P.D. with increasing concentration. With increasing concentrations of these salts the negative P.D. is reduced to zero, becomes positive, rises to a maximum positive P.D. and then falls to a zero P.D. again when the salt concentration attains a value of about 10^{-1} to $10^{-0.5}$ equivalents per liter. Similarly, with HCl, HNO_3, H_2SO_4 and picric acid, the P.D. on *Bact. typhosum* is decreased from its usual negative value of about 40 millivolts to a 0, using 10^{-7} to 10.0 equivalents of acid per liter.

* *See also* Stearn and Stearn, *J. Bact.,* 9, 463, 479 (1924).

With HCl and HNO$_3$ (and somewhat with H$_2$SO$_4$) there is a reversal of P.D. to $+$ value, these positive values reach a maximum and then decline again to zero P.D. Picric acid does not bring about a reversal of charge. At pH 2.0 the P.D. of $+$ 12 millivolts on *Bact. typhosum* is diminished by salts in the following order of effectiveness: Na, Mg, Ca. La chlorides $<$ Na$_2$SO$_4$. On the bacillus of rabbit septicemia (Type D) (no pH controls) the order of increasing effectiveness in reducing and reversing P.D. is:

$$NaCl, Na_2SO_4 < CaCl_2, BaCl_2 < AlCl_3 < HCl < Th(NO_3)_4$$
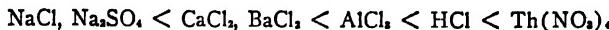
from 10^{-7} to 1.0 equivalents per liter. This indicates that the effectiveness of the ion, whose charge is opposite to that of the bacterium in modifying P.D., increases with its valency. At pH 3.0, with increasing concentration of salt (NaCl and Na$_2$SO$_4$) the P.D. on Type D, starting from 0, becomes increasingly more negative; AlCl$_3$ first brings the P.D. to positive values and then back to zero. At pH 2.0, the order of increasing effectiveness in shifting $+$ P.D. to 0 or to $-$ P.D. is: Na$_2$SO$_4$ $>$ AgNO$_3$ $>$ AlCl$_3$, BaCl$_2$, NaCl.

The Type D strain of rabbit septicemia organism is more electronegative ($-$ 30 mv. at pH 7.0) than the Type G strain ($-$ 20 mv. at pH 7.0) and is correspondingly more difficult to agglutinate. Or, conversely, the Type G strain is more commonly spontaneously agglutinable.

Northrop and DeKruif (1922) describe a method of measuring the cohesive force between bacteria—measuring the force required to pull apart two heated (60°) films of bacteria on glass immersed in the test fluid. The effects of electrolytes on cohesive force as measured by this method begins to be evident when the concentration becomes 0.1 N or greater. The effectiveness of various electrolytes (NaCl, LaCl$_3$, HCl, Cu acetate, MgCl$_2$) appears to be independent of the valency or the electrical effects of the ions. LaCl$_3$ is more effective than NaCl in reducing potential, but is less effective in reducing the cohesive forces.

These experiments of Northrop and DeKruif show what has usually been obtained by other experimenters, that a low concentration of salt precipitates bacteria and that higher concentrations stabilize their suspension. "They also show that this is due in most cases to the fact that excess salt or acid confers a high potential of opposite sign to that in low concentration." Another observation which is in harmony with the conclusion of Beniasch (1912), Putter (1921), etc., is that the effectiveness of an electrolyte in *reversing* the P.D. depends on the nature of the suspension. Thus, NaCl and Na$_2$SO$_4$ will *reverse* the charge on the bacillus of rabbit septicemia but will only *reduce* the charge on *Bact. typhosum*. They found that immune serum displaces the isoelectric point of bacteria in the alkaline direction and that peptone shifts the charge and displaces the isoelectric point in the acid direction. The significance of these experiments of Northrop and DeKruif may be summarized in their own words:

". . . it has been found that whenever the potential difference between the surface of the bacteria and the solution is less than about 15 millivolts the bacteria agglutinate, *provided the cohesive force is not affected*. If the cohesive force is decreased, this critical potential is decreased, and if the cohesive force is made very small, no agglutination occurs even though the potential be reduced to zero. It was further found that all electrolytes tested in concentrations less than 0.01 to 0.1 N affect primarily the potential, while in concentrations greater than 0.1 N the effect is principally on the cohesive force. In the case of bacteria sensitized with immune serum, the cohesive force remains constant and the agglutination can be predicted solely from the measurements of the potential." (P. 641.)

Northrop and DeKruif (1922, a) showed that the addition of albumin to suspensions of rabbit septicemia organisms (Type D) shifts the isoelectric point (pH of zero P.D.) of the bacteria towards the isoelectric point of the protein (i.e., towards pH 5.0 from pH 3.0); the addition of globulin shifts the isoelectric point towards pH $= 6.5$. Normal and immune sera both shift the isoelectric point towards pH $= 4.5$. As Krumwiede and Pratt (1913), Michaelis and Davidsohn (1912), Eggerth and Bellows (1922) and others had found, the amount of immune serum necessary to agglutinate bacteria is minimal at or near the isoelectric point. Agglutination by immune and normal serum, as by acid and electrolytes, occurs when the P.D. fails to exceed 15 millivolts. This value checks very closely with the values calculated above from the data of Girard and Audubert (1918) and with the value intimated by the experiments of Shearer (1922). Bordet had shown that salts increased the agglutinating potency of immune sera; Porges (1905) showed that with very powerful immune serum agglutination occurred even though the serum had been dialysed free from salt. In these studies of Northrop and DeKruif (1922, a) dialysed sera, immune or normal (taken up with a trace of NaOH—total salt concentration less than 0.001 N) did not cause complete agglutination although both reduced the P.D. When salt is present, its potency to cause agglutination through changes of the P.D. is, within bounds, additive with that of the immune body. It appears that serum in the presence of concentrated salt solution primarily affects the cohesive force, increases it and causes agglutination. For example, the cohesive force may be raised to its value for distilled water suspensions of bacteria by immune serum despite the presence of 0.10 N concentration of salt. On the other hand, NaCl up to 8.0 N has practically no influence on the cohesive force of *Bact. typhosum* sensitized with immune serum, although a high salt concentration ordinarily reduces the cohesive force between normal, unsensitized bacteria. The agglutination—in the presence of the immune body—may be considered, as Bordet inclined to think, due to the salt rather than to the immune body. The experiments of Northrop and DeKruif (1922, a) also indicate that when dealing with electro-negatively charged organisms, the valency and nature of the anion of an electrolyte are inconsequential. On the other hand, although monovalent cations all behave much alike and the divalent cations behave much alike, the latter are more effective than the former in reducing the P.D. on sensitized organisms. The following series of efficacy in this respect may be arranged from their data:

$$\left.\begin{array}{l} NaNO_3 \\ NaCl \\ NaOH \\ Na\ Acetate \\ Na_2SO_4 \\ KCl \\ LiBr \end{array}\right\} \quad < \quad \left.\begin{array}{l} CaCl_2 \\ BaCl_2 \\ ZnSO_4 \end{array}\right\} \quad < \quad Cu\ acetate \quad < \quad \left.\begin{array}{l} H_2SO_4 \\ HCl \\ HgCl_2 \end{array}\right\} \quad < \quad AlCl_3$$
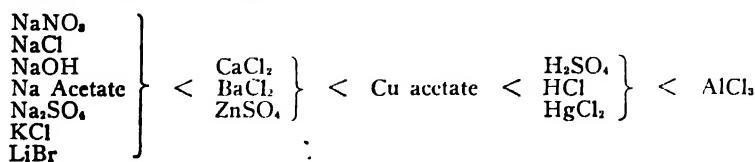
The bearing of these experiments on the mechanism of the fixation of immune body upon antigen and upon agglutination is not, however, as simple as might appear at first sight. According to DeKruif and Northrop (1922) it appears true that the amount of immune body necessary to cause agglutination of *Bact. typhosum* varies directly with the concentration of suspension. But the amount of immune body which is combined with the bacteria is independent (i.e., is constant) in the pH zone 3.7 to 9.0 where the P.D. is in-

creasing steadily with pH. Below pH = 3.7 the amount in combination is decreased. At pH = 2.5 immune serum increases the + P.D. of the suspended bacteria.

"These results are contradictory to the idea that the combination is caused by a difference in the sign of the charge carried by the immune body and the organism. They agree with the assumption that the immune body forms a film at the surface of the organism and that the effect on the charge is the result of this film." (DeKruif and Northrop, 1922, p. 137.)

The constancy over a broad pH zone of the amount of immune body which combines with the electro-negative bacteria—a zone of pH over which P.D. increases but not at all in proportion to increases in pH—proves, to our mind, merely that the combining power of immune body for organism is independent of the *magnitude* of the electro-negativity within the precision of the technique but not of the negativity itself. The uncertainty that the nature of the charge on immune body reverses with pH is clearly recognized by the authors. We incline to doubt that the evidence is incontrovertibly contradictory to the idea that the combination of immune body and bacterium is caused by a difference in the sign of the charges which they carry. On the contrary, much of the evidence adduced by DeKruif and Northrop may be interpreted in favor of such a view.

In the following paper, DeKruif and Northrop (1922, a) point out that when immune body is fixed to bacteria the zone of acid agglutination is widened. Hence they show that the width of the acid agglutination zone can be used as a method of testing for the presence of immune body. And in a more recent paper (DeKruif and Northrop, 1923) they report that unstable (spontaneously agglutinable) suspensions of rabbit septicemia organisms (Type G) can be rendered stable by adjustment of pH to 7.0-7.5. Five intensely auto-agglutinable strains of *Streptococcus haemolyticus* were rendered stable by suspension in 0.001 N NaOH. If the serum dilutions are made in dilute (M/320) NaCl solution, these alkaline stabilized suspensions can be used to demonstrate the specific action of rabbit antistreptococcus serum.

Throughout this review of the literature on the electrical charges on bacteria we have not attempted a systematic review of that closely related field, the rôle of electrolytes in specific and non-specific agglutination of bacteria. The Presidential Address of Buchanan (1919) presents an entirely adequate compilation and digest of that literature, as well as some interesting new data which take on a new significance in the light of the contributions of Northrop and DeKruif. It is not beside the point to quote the following from his Address:

"It is evident then, that we have at least a partially adequate explanation of why bacteria under certain conditions remain in suspension, and why under other conditions they may cling together. We may regard the similar electric charge as constituting the repulsing agency, and surface tension as the attracting agency. A study of the agglutination phenomenon then resolves itself into a consideration of the means whereby these two forces may be modified, increased or diminished. Agglutination occurs whenever the similar electric charges are decreased to amounts such that they will no longer overcome the pull of surface tension. Or conversely, surface tension may be increased until it overcomes the dispersion effect of the similar charges. . . ." (Page 78.)

Later he propounds the question: "It may be accepted then that the presence of electrolytes may modify the charge on a bacterial cell. But how?" His answer to the question is given in the following terms:

"Apparently by the adsorption of ions or particles bearing an opposite charge. Conversely, the charge on the cell may be due to the various substances adsorbed, frequently to the ions. It is necessary that there be such an affinity between the positive cation, for example, and the negative bacterial cell that the latter will adsorb the former. The dispersive force is thus gradually neutralized." (Page 79.)

Significant contributions which supplement the bibliography of Buchanan (1919), besides those which have already received mention are the papers by Dochez, Avery and Lancefield (1919); Eisenberg (1919); Bordet (1920); Arkwright (1921); Kosaka and Seki (1921); Mellon (1922); Mudd (1923) and Mudd and Mudd (1923). The paper of Mellon (1922) brings up the question of ionic antagonism which has not yet received the attention which it deserves.

A few conclusions from this incomplete review of the more extensive body of literature which has been consulted seem clear:

1. The evidence seems overwhelming that in neutral and in slightly acid or slightly alkaline media live or dead bacteria commonly carry a negative charge (i.e., migrate to the anode in an electrical field).

2. The yeasts, it appears, probably carry the same sort of charge as do the bacteria.

3. The spirochaetes and protozoa appear to be divided between strains or species which bear positive and negative charges. This needs confirmation.

4. The addition of acids or other electrolytes to electronegative bacteria results in a diminution in their electronegativity.

5. The isoelectric point of bacteria in water, i.e., the pH of zero electrical potential difference between them and their menstrua, is commonly near pH 3.

6. The acidulation of media beyond the isoelectric point of the bacteria may or may not result in the development of a positive charge on the bacteria. This seems to depend as much upon the nature of the organisms as upon the nature of the reagents employed.

7. The efficacy of electrolytes in reducing the charge on electronegative bacteria or increasing the charge on electro-positive bacteria depends upon the concentration of the electrolyte and upon the valency and nature of the cation.

8. The efficacy of electrolytes in reducing the charge of electro-positive bacteria depends upon the concentration of electrolyte and the valency and nature of the anion.

9. Bacteria are commonly agglutinated when the P.D. between them and their menstruum falls below a value of about 15 millivolts. This critical charge is much less for certain groups of bacteria. (Cf. references in *addenda.*)

10. Electrolytes in small concentrations appear to affect the stability of suspensions of bacteria by modifying the P.D. between bacterium and fluid. In high concentrations their effect upon the stability of suspensions appears to be associated with changes in the cohesive forces between the dispersed bodies.

11. Autoagglutinable suspensions may be rendered stable by decreasing the cohesive forces between the bacteria or by increasing the charge on them. The latter may commonly be accomplished either by shifting the pH away from the isoelectric point or by adding electrolytes which will give polyvalent ions of the same charge as the bacteria.

12. Stable suspensions of bacteria may be rendered more unstable or

spontaneously agglutinable by shifting the pH towards the isoelectric point or by adding electrolytes, particularly those which give polyvalent ions whose charge is opposite to that on the bacteria.

13. The growth and viability of bacteria are probably closely associated with their electrical condition.

More recently, Winslow, Falk and Caulfield (1923) have confirmed many of the findings of Northrop and DeKruif in regard to the following points:

(a) The general tendency of the bacterial cell, when suspended in distilled water near the zone of neutrality, to move toward the anode of an electrical field;

(b) The fact that the migration of bacterial cells is a function of the [H+] of the menstruum. The curve obtained by plotting velocity of migration against pH passes through an isoelectric point at about pH = 3; at greater acidity the direction of migration becomes reversed (toward the cathode); and in still more acid solution (pH = 1) again disappears; while at reactions less acid than pH = 3 the velocity toward the anode increases with increasing alkalinity.

(c) The fact that neutral salts depress the velocity of migration, calcium chloride being much more effective than sodium chloride in the same concentration.

They found further that:

(a) On the extreme alkaline side of the curve of velocity of migration plotted against pH, a maximum value is reached at about pH 10 (for *Bacillus cereus*) with a fall at about pH = 12. In many experiments an isopotential point is reached at a suitable alkalinity.

(b) The depressing effect of salts is accompanied by a general shifting of the curve of migration velocity so that a maximum velocity appears at about pH = 7 and an abolition of velocity at about pH = 9 to 10.

(c) An apparent "antagonistic" effect is indicated between CaCl$_2$ and NaCl, the presence of a certain concentration of the latter salt diminishing to a slight but definite degree the depressing effect produced by the former.

(d) Heat killed bacterial cells exhibit essentially the same curve of migration velocity as that of living cells.

(e) Bacterial spores exhibit the same general curve of migration velocity as vegetative cells, although the actual velocity is apparently slightly less.

The existence of an alkaline as well as an acidic isoelectric point has been confirmed by Winslow and Shaughnessy (1924) working with *Bact. coli*. The effects of a number of salts, proteins, sera, etc., upon the electrophoretic potentials of bacteria have been studied by Eggerth (1923), Shibley (1924) and others. For a further discussion of the origin of the P.D. and its rôle in determining bacterial or colloidal stability, we may refer to the review by Northrop (1924).

In reports on recent experiments with pneumococci, Falk, Gussin and Jacobson * have studied at length the relations between electrophoretic potential and microbic virulence for experimental animals. They have demonstrated a direct parallelism between these two characteristics for pneumococci of Types I, II, III and IV and for certain variant strains of Type I.

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ADDENDA

Some important new advances are reviewed in the following publications:

"The Newer Knowledge of Bacteriology and Immunology," edited by E. O. Jordan and I. S. Falk (Chicago, 1928)—see papers by I. S. Falk on "A theory of microbic virulence," by J. H. Northrop on "The mechanism of agglutination," and by W. D. Harkins, on "Hydrogen-ion concentration."

"Hydrogen-Ion Concentration," by Leonor Michaelis; trans. by W. A. Perlzweig, Vol. I, 299 pp. (Baltimore, 1926).

Colloid Chemical Problems of Serology

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I. Introduction

For years various attempts have been made to explain certain phenomena in the study of immunity, by relating them to known physico-chemical phenomena. In this manner, the nature of the mechanism of the immunity reaction [3] has been extensively studied. Ehrlich [16] believed that in the serological reaction, e.g., in toxin-antitoxin fixation, a chemical process takes place. The difference between Ehrlich's and Arrhenius' point of view concerning this question, was only that Ehrlich conceived a practically complete reaction with an unusually great affinity-constant, while Arrhenius assumed a weak affinity; that is, a considerable hydrolysis, and also the presence of a fourth substance. Arrhenius [1] succeeded in computing the quantitative relation in the toxin-antitoxin fixation, on the basis of the law of mass action, in which he chose arbitrarily the stoichiometric relations and the affinity constants. The conformity with experiment was very good.

It was still the tendency as that time, according to the law of mass action, to regard the reaction in question as a homogeneous chemical reaction. It should be considered, however, that particles, perhaps even those microscopically visible, are subject to the same laws as molecules. (See Perrin: [46] The Atom; Gyémánt; [24] Linderstrom-Lang. [37]) They are capable of reaction (surface reactions), and their active mass is in proportion to the number of particles. Accordingly, surface reactions in microheterogeneous systems obey the laws of mass action, as instanced in the well-known law of adsorption of Freundlich. [20]

In fact, some years earlier (1904), it had been shown by Biltz, [6] Eisenberg and Volk, [17] that antigen-antibody neutralization curves, with suitable choice of constants, are represented by the Freundlich adsorption curve. This was a reason, among others, for emphasizing the point that immunity reactions result from the interaction of two colloids (Bordet, [8] Landsteiner, [38] Zangger, [61] and others). According to this, antigen and antibody must be colloid substances. There is no lack of facts which appear to prove that this is true. Investigations of diffusion (Arrhenius and Madsden; [2] Flexner and Noguchi [18]), dialysis (Pick [48]), ultrafiltration (Bechhold [5]) of immune substances, showed that they behave as colloids and resemble proteins. Also, their adsorbability (Landsteiner [31] and Bechhold [4]), their behavior in an electrical field (Landsteiner and Pauli [32]), sensitivity to heat, precipitation by salts (Pick [47]), ageing and inactivation by shaking (Marie and Tiefanau [30]), all sustain this theory.

Based upon these facts there arose a colloid-chemical theory of serum

reactions, according to which they follow the laws of adsorption. The adsorption is specific; at first reversible, and becoming irreversible only by means of secondary processes. According to Bordet,[7] serum reactions are differentiated from those between dyes and substrates only by their unusually high degree of specificity.

However, the cause underlying the immunological specificity of individual colloids, e.g., proteins, we find that this is not to be explained by their known colloid-chemical characteristics. It is scarcely possible to distinguish between denatured and native human serum albumin immunologically, though it is easy to distinguish human from horse serum albumin. Landsteiner,[34] who was one of the first to recognize the importance of the colloid state, also first studied the influence of the chemical constitution on the serological specificity by means of his artificial azoproteins. Combinations of albumin and substances of known chemical constitution, produced by azoconjugation, e.g., of metanilic acid, aminobenzoic acid, etc., manifest a specificity which is entirely dependent on the group with known constitution, and scarcely at all on the proteins. Landsteiner was also able to demonstrate in these tests, that the stereochemical configuration is of special significance; that substances which are chemically related, are also serologically related. Landsteiner designated as *haptens* the groups which determine specificity (component parts of antigens). The haptens react with the immune body; they are, however, not capable of stimulating the formation of immune bodies in the animal organism. Such haptens were recovered from alcoholic extracts of animal cells (the heterophile hapten—Forssman[19]) and from bacteria (Sordelli and Fischer,[54] Heidelberger,[28] Avery,[3] and Zinsser and Parker,[63] Landsteiner and Levene[35]). Here also it was demonstrable that the chemical constitution is of great significance.

These results have turned the further investigation strongly in the direction of chemical constitution. In addition to this, research on the related subject of ferments followed the same course (Willstätter[60] and associates). The rôle of the colloids has been the field of much discussion and doubt. This is particularly due to the fact that for the explanation of serological phenomena, there were advanced colloid-chemical or capillary concepts which are not clear, and of which much less is known, perhaps, than of the serological reactions themselves.

Serological phenomena cannot be entirely explained by means of the present knowledge of colloids. It cannot, however, be doubted that they are closely connected with changes in the colloid system. If serological phenomena are considered from the standpoint of colloid chemistry, we discover interesting problems, perhaps the most unusual being that of reciprocal flocculation, independent of the charge. It would be an error to regard the colloid state as an accidental characteristic of a substance or system, independent of other physical and chemical conditions; it is closely related to other properties and the chemical constitution of the substance. Between the chemical and physical points of view, referred to above, there is no contradiction. We should rather attempt to become acquainted with the previously unknown behavior of certain colloids, which can explain the specificity which is so characteristic of serological reactions. This is not only an important problem in serology, but also of interest for colloid chemistry.*

* When particles aggregate, the contour of the residual electric fields of the aggregates will, generally, be quite different from those of the particles forming the aggregate; and the kinetic activity of the aggregates is diminished. Whatever may have been the immediate cause of the aggregation, these two

II. The Colloid Nature of Antigens

The earlier work already mentioned concerning the physical characteristics of antigens was carried out with native instead of purified products. Thus, the objection may be made that the antigen was associated with colloids and therefore partook of the characteristics of the latter. It has not been possible to prepare a chemically well-defined, pure antigen from most of the natural antigens. The purest and best known antigens are glucosides from fungi; crystallized egg albumin and hemoglobin. (Sörenson,[55] for serum albumin, by means of solubility, and Doerr and Berger [13] by immuno-chemical methods, have proved that they are not single substances.) These relatively pure antigens are colloids. It appears, therefore, that the colloid state is a necessary condition for the exercise of antigenic function. On the other hand, only relatively few colloids possess antigenic properties. Other characteristics of these substances must play some part, as is also seen in the experiments of Landsteiner, which have been already mentioned, and in analogies between idiosyncrasy and anaphylaxis. From Landsteiner's results we know that it is possible to change the specificity of an antigen, or to bring about a new specific quality, by the addition of a known, relatively simple compound, and substances which show similar behavior (haptens) can be prepared from natural antigens (Heidelberger and Avery [28]) so that it may be said in general that antigens must possess hapten groups. From this point of view, quinine, antipyrine, and other drugs which call forth idiosyncrasies, may be looked upon as haptens (Doerr [12]).

We have seen that haptens bring about the union of antigen and antibody; that is, react with the antibodies. They must also, therefore, play a definite rôle in the formation of the antibody. Concerning this, however, almost nothing is definitely known. Besides, the class of substances to which the haptens belong is difficult to define. It is apparent, however, that they are often cyclic combinations. The "ionogen" (acid) group was inclusively recognized by Landsteiner, as a hapten particularly specific in action.

It seems as though the haptens were carriers of every quality peculiar to antigens. In what, then, does the rôle of the colloid system consist? According to Zinsser [62] it prevents the penetration of the antigen into the cells, and so these are forced to secrete antibodies. To this end, any colloid, whether inorganic or not, would be capable of being loaded with haptens. At the present time, however, no experiments are known to prove that non-antigenic colloids are capable of influencing haptens to antigenic activity. The only exception which I know of is an experiment by Doerr and Hallauer,[14] according to which it is possible to secure hemolytic sera by the injection of homologous red blood corpuscles with heterophile haptens. It is worthy of notice that in certain cases (lipoid haptens), the addition of the haptens exerts no influence whatever on the specificity of the residue. By this addition, the residue takes on a new character, entirely independent of the original antigen; it functions as a double antigen.

Probably haptens influence colloids in some manner which is at present unknown, and it is just by this means that they exercise their activity. This influence cannot, however, involve an alteration of the degree of dispersion, or of the hydration, or the charge of the colloids, as has often been suggested;

consequences may exert a profound influence on the nature and velocity of the adsorbability or reactivity of the aggregates as compared with their component particles. *J. A.*

for we know that such alterations of antigens do not influence them at all qualitatively, and very little in their degree of activity. There are colloid qualities concerned here, apparently, which as yet have scarcely been studied. The attention of colloid chemists will be directed to these through serology and specific biologic phenomena.

III. The Existence and Colloid Nature of Antibodies

It has already been mentioned, that antibodies diffuse with difficulty; that they are colloid substances which are held back in dialysis and in ultrafiltration. And here the objection may be raised that these experiments have not been made with pure materials, and the antibodies were in some manner bound to colloids. Experiments to purify materials have led to even less definite results here, than with antigens.

The non-dialyzable fraction of an immune serum consists of albuminous material, and of lipins which are soluble in alcohol and ether. By fractional precipitation with ammonium sulfate, or by dialysis, the euglobulin, pseudoglobulin and albumin can be separated from serum protein.

Based on the experiments of E. P. Pick,[47] Banzhaf and Gibson,[3] and others, it is to-day generally accepted that antitoxin is associated with pseudoglobulin, and some other antibodies with the euglobulin.* Among the earlier experiments some are found (Gloor and Klinger;[22] Kapsenberg[30] and Stern[57]), in which luetic antibodies were present in both fractions, or only in the euglobulin fraction. More recently Doerr and Hallauer[15] found the anaphylactic immune body in the euglobulin, as well as in the pseudoglobulin-fraction, while Otto and Shirikawa[41] found it in the euglobulin fraction.

These contradictory results are to be explained in part by the fact that serum fractions are not well defined; their composition varies greatly, if the mode of preparation is changed only slightly. The luetic antibody is found partly in the pseudoglobulin and partly in the euglobulin fraction if undiluted serum is used, and quantitatively in the euglobulin fraction, if diluted serum is used.

It is possible to consider this peculiar behavior of the globulin as due to a change in its precipitability due to the presence of lipins ** (H. Chick,[10] Jarisch,[20] Matsumura,[40] Hartley,[27] etc.).

If immune sera are extracted with ether and alcohol (Hardy and Gardiner[25]) we find that in the case of antitoxic sera (Hardy and Gardiner, Hartley,[27] and others), the antibody is present in the water-soluble residue. Precipitating sera (Hartley, Reiner and Kopp[51]) and also sera which contain the heterophile antibody behave in a similar manner. According to Hartley, Wassermann-positive sera become negative for the most part, after extraction. This could not be confirmed by Reiner and Török,[52] who found merely that the Wassermann reaction was somewhat weaker after extraction. Friedberger[21] and his co-workers found that heterophile precipitins disappear from immune serum after extraction with ether. This also could not be confirmed. In the lipin fraction of the serum, Reiner and Kopp were unable to demonstrate heterophile antibody. It was found, however, in the extracted serum.

If fractionation of extracted immune serum is carried further, either by electrodialysis or by the salting out method, there is usually found a decrease

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* It should be noted that the sera of different animals act quite differently in this relation. See Pick.
** Colloidal protection (often cumulative protection, see Vol. I) seems to be a phenomenon generally present in cases of this kind. *J. A.*

of the titer. (Reiner and coworkers.) Doerr and Hallauer observe an increase of the hemolysin content, if they subject a non-extracted serum to electrodialysis. The antibody is found most often in the euglobulin fraction, or in both the euglobulin and the pseudoglobulin. On electrodialysis of a globulin solution from an extracted serum, we find that at the end of electrodialysis, a great part of the antibody (hemolysin, precipitin) remains in the solution. Its behavior is that of the so-called pseudoglobulin.

Experiments of Sörenson,[56] as well as others,[49] with extracted serum, show that the globulin fractions which can be separated from serum, are mixtures of a water-soluble (pseudoglobulin) and a water insoluble (euglobulin) protein. If the antibodies were present in only one of the fractions, e.g., in the precipitate after electrodialysis, even this would be no proof that they are associated with the euglobulin, and still less that they are controlled by the colloid state of the globulin. It is, however, easy to show that this precipitate contains a part of the so-called pseudoglobulin.

In serum there are substances which hold in solution certain proteins (globulins) which react with these substances, though apparently not in stoichiometrical proportions; expressed in terms of colloid chemistry these substances exert a peptizing action on proteins. Accordingly, how much albumin is contained in solution would depend on the relative amount of the peptizing medium. This leads to the phenomenon, paradoxical from a physico-chemical standpoint, that less globulin can be separated from concentrated than from dilute serum.*

Globulin from extracted sera behaves exactly as does globulin from native serum. The globulins are therefore easily separated from the lipins as are also the antibodies. The view held by some authors (Sachs [58] and others; see Wells [59]) especially in relation to the much studied luetic antibody, that antibodies are affected by the physical state of the globulin and by increase of the lipins, must be considered as erroneous. By such influences, individual reactions are secondarily strengthened, but this has nothing to do with specific antigen-antibody fixation. This is shown not only by the before-mentioned decrease of titer after extraction and fractionation, but also by the fact that the activity of immune serum (precipitating serum) can be restored, if to the precipitate obtained by electrodialysis is added the residue and a corresponding amount of sodium chloride, and the mixture shaken for several hours. Doubtless, this proves that the occurrence of a serological reaction is very markedly influenced by the physical condition of the medium. However, the physical structure—the precipitability of the globulin is not the specific factor, as is shown by the fact that an antibody may be removed quantatively from an immune serum (hemolytic), without actually influencing the composition of the serum itself. The albumin-globulin quotient is not notably changed after this, which would necessarily be the case if antibody containing globulin had been adsorbed by this method.

The significance of serum-colloid for antibody function, is similar to the significance of the colloid state for antigen function. If there is a change in the physical colloid structure of the serum—the so-called globulin solution —then the antibody function disappears. Antibody function can be preserved by specific adsorption, without actually influencing the structure and composition of the serum. There must, however, be specific reacting bodies in the

* This apparent paradox may be perhaps due to the fact that protective colloidal action does not parallel concentration, becoming marked only at a certain concentration in the cumulative protective chain. See paper on colloidal protection by J. Alexander in Vol. I of this series. *J. A.*

serum, which apparently are loosely combined with the hydrophobe albuminous substance (globulin); so far they are analogous to the haptens and may be called *immune haptens*. It must be emphasized, however, that these bodies have thus far not been isolated. Their existence can be inferred, however, with some degree of certainty.

IV. On the Mechanism of Fixation and Future Outlook

It was mentioned in the introduction, that the quantitative course of immunity reactions (toxin-antitoxin fixation; agglutinin fixation) does not definitely determine by what mechanism these reactions take place. Many much-discussed analogies, however, suggest that the reaction is the same as that of the interaction (flocculation) of two colloids, concerning which latter we also know very little. With most colloids, particularly with inorganic colloids, the nature of the electrical charge appears to exert particular influence. The reciprocal flocculation of two colloids occurs most frequently when they are oppositely charged. Most colloids flocculate more readily the smaller the electrokinetic potential,* which depends upon the charge. For the stability of colloid solutions a minimal potential is necessary. The amount of this minimal potential depends partly upon the character of the chemical constitution of the colloid, and partly on the medium. Flocculation can be caused by discharge as well as by a chemical change in the colloid. Such a change in aqueous dispersions is known as dehydration. The chemical reactivity—peptization by chemical means—has been studied in inorganic colloids, principally from the standpoint of ionization, by Zsigmondy,[64] Pauli,[45] ** and their co-workers. Organic colloids, however, can be peptized also by non-ionizing substances; i.e., by reactions with non-electrolytes. Thus, for example, the flocculation of albumin is altered by formaldehyde. The chemical reaction here is apparently the addition of formaldehyde to the amino groups (Reiner and Marton [50]).

In the reaction between antigen and antibody there is no question of a discharge process. Michaelis and Davidsohn,[41] and recently Coulter,[11] have demonstrated that the course of this reaction is entirely independent of whether the charge is like or opposite. Northrop [42] and his co-workers [43] have deduced from the experiments of Bechhold [4] that the fixation of the agglutin by an antigen is not accompanied by a decrease of the electrokinetic potential. In order to prevent flocculation, the charge of the antigen must be increased. We are here dealing with a chemical and not an electrochemical process (dehydration), which has been little studied by colloid chemists, and which greatly decreases the stability of the suspension (bacteria or cells). From the experiments of Stern,[57] we presume that all antigen-antibody reactions take place with dehydration. They lead to complement fixation just as in the case of artificially produced dehydration. Also, the well-known tendency to precipitation of the antigen-antibody complex, must be considered as a consequence of dehydration, since, according to Northrop, it is not a matter of discharge.

But how does this dehydration occur? This is the real colloid chemical problem of serology. Its answer must, at the same time, explain the question of specificity. Specific fixation and dehydration always parallel immunity reactions. It has often been claimed that colloid chemistry cannot explain

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serological phenomena, since it does not explain specificity. This *"ignorami-bus"* can be asserted only if one believes that colloid chemical phenomena can be fully explained by simple processes of charge and discharge. This is, however, not the case, especially in serological reactions.

We have already seen that the specificity which is essential in serological reactions is not determined by the colloid state, but by well-defined elements in the molecular structure. We have also seen that these substances alone do not show serological reactions. Since specificity is conditioned by them, specific dehydration must also depend upon them. In order to make this problem clearer, their ability to react, if they are bound to colloids, must be investigated further. First, however, in order to develop a point of view let us consider a few observations.

Since antigen and antibody retain their specific biological characteristics even when combined, and manifest no new ones, we may conclude that the reaction which underlies fixation, in the continuous series between purely chemical reactions and purely physical adhesion, more nearly approaches the latter. Then, in the case of a stable condition, the adhesion of the particles to the medium (electrolyte solution) is greater than the cohesion of the particles.*

Two colloids form a stable complex in case:

$$A_{koll._1 - koll._2} = \gamma_{koll._1} + \gamma_{koll._2} - \gamma_{koll._1 - koll._2} > \begin{cases} A_{koll._1} \\ A_{koll._2} \end{cases}$$

$A_{koll._1 - koll._2}$ must be greater, if a specific reaction between 1 and 2 is concerned, than with any other combination of 1 with n or of 2 with n. However, since $\gamma_{koll.}$, in the various biological colloids which come into consideration, is of about the same order of magnitude, the ability of two stable colloids to react depends essentially on the relation of their interfacial tensions.**

Harkins [26] and Langmuir [36] have investigated the interfacial tension between two immiscible fluids, and found that it depends upon the structure and composition of the molecules in these fluids. Toward water, which of itself is strongly heteropolar, especially heteropolar combinations, e.g., alcohols and aldehydes, show a strong adhesion. But among these also there are wide differences, which are caused by differences in the molecule. The adhesion, and the interfacial tension also, therefore, depend on molecular forces (residual valencies or electrostatic residual fields). The importance of this conclusion lies in the recognition that these forces are directed forces, or better expressed, that the fields of force are not homogeneous, and that the structure of the field of force in the immediate neighborhood of the particle depends upon the arrangement of the positive and negative charges in the molecule. Thus, there arise on interfaces between fluids, structures which are definitely related to the molecule. The differences in specific adhesion between two fluids are, according to the investigations of Harkins,[1] not very great; they can, however, become significantly greater if substances with high surface tensions are investigated—solids, for example. Most colloids which come into question

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* The significance of the adhesion (the interfacial tension) in agglutination (that is, the precipitation of the antigen united with antibody), has been studied by Buchanan and by Green and Halverson. In the following, however, the role of adhesion in the primary process antigen antibody fixation, will be considered.

$$A_{koll. - med.} = \gamma_{koll.} + \gamma_{med.} - \gamma_{koll. - med.} > \begin{cases} 2\gamma_{koll.} \\ 2\gamma_{med.} \end{cases}$$

where γ signifies the free surface energy per surface unit.

** See paper by J. Traube in Vol. I of this series. *J. A.*

here are to be so considered. (In solids γ koll. will be great, and therefore with γ koll.₁ — koll.₂ the adhesion will be great.)

In order to show the existence of a specific adhesion, it seems pertinent to begin with the Langmuir-Harkins experiments.* It is, however, easy to understand without these, that on the surface of these solid particles there are active forces, which depend on the constitution—in a stereochemical sense— of the molecule or molecular groups found on the surface. (Weissenberg [58] called such groups *dynads*.) They are responsible for both crystallization and adsorption. Concerning the nature of these forces, we know only that they are brought about by partially saturated electrical charges (dipolar) or groups of charges; they should be really active only in the closest proximity to the surface (10^{-8} cm.), and beyond this range become inactive rather abruptly. This is actually the case in the gaseous state. They are propagated by intramolecular polarization. (See the adsorption theory of Lorenz and Landé.[88])*

The influence of chemical and stereochemical constitution upon the reactivity of colloids is not only explained by these facts, but actually must be assumed; and it is not at all justifiable to assert that the specificity of the serological reaction and of biological phenomena in general, is incompatible with the colloid-chemical viewpoint. All possible surface structures may be divided into *"banal"* and *"complicated."* "Banal" structures are incapable of specific reaction. There is a much greater similarity among them than among complicated structures**

Among antigens the complicated surface structure is brought about by haptens. And similar bodies which determine structure must be assumed among antibodies. These structures are, however, very much smaller than those which could be formed by colloid particles. Elements of such structures are apparently repeated many times on a globulin surface. By the adsorption of the haptens, the surface energy of the colloids is changed to one which is characteristic for the haptens. That this energy actually exists is due to the colloid state.

The essential phenomena of serological reactions are easily explained by the analogy briefly sketched here. The conception is, however, remarkable also from the colloid chemical standpoint, in that it shows that one can anticipate, on the ground of present physical knowledge, that colloids have another important characteristic beside those already studied, by which they may be differentiated from each other; this being the structure of the surface in the stereochemical sense.

Specific fixations play an important rôle not only in the study of immunity, but also in the study of ferments and of inheritance. Apparently these fixations have a similar mechanism. Perhaps it is not too much to say that the

* See papers by W. D. Harkins, and I Langmuir in Vol. I of this series. *J. A.*
* The propagation and stabilization of the structure of the haptens possibly may explain the significance of the colloid state for antigen function.
** "Banal" and "complicated" are vague concepts. In order to illustrate what is meant thereby, let us think of a chess board, on which the problem is to find surfaces made up of the elementary fields with which the chess board can be covered, so that the areas which can be covered by corresponding colors shall be as large as possible. It is easy to see that it is possible to arrange a large number of surfaces in which the primary fields, alternately black and white, form right triangles, and which would so cover one-half of the chess board that like colors would overlie each other. However, if we take a mosaic which presents a complicated surface picture, with as uniform a color distribution as possible, it would then be difficult to find a second mosaic with which the picture could be so covered that even one-hundredth of the total surface would be covered by its corresponding colors. A mosaic suited to an extensive covering with the same colors cannot be found, but must be constructed (most easily by mirror reflection). (See paper by Alexander and Bridges in this volume. In serology this indicates that an antibody can form only through direct influence of an antigen. *J. A.*)

characteristic determinant of heredity must also be the peculiarity of certain bio-colloids, since its power of reaction, and consequently its further course, is influenced by the complex structure of its surface.

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Serum Diagnosis of Syphilis

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I. INTRODUCTION

There are few laboratory methods in medical practice which offer more
problems to the colloid chemist than the Wassermann and precipitation tests
for syphilis. On the one side, the physico-chemical mechanism of these reac-
tions is obscure. On the other side, many aspects of their clinical inter-
pretation are unknown. Furthermore, these tests are usually classified among
immunity reactions, although there is far from ample proof that this classifi-
cation is correct. It will be recalled that in immunity reactions some substance
of protein character such as bacteria, etc., referred to as *antigen*, is introduced
into an animal, and as a result there are produced in the animal *antibodies*
which can combine specifically with the antigen. In syphilis, on the other
hand, although the *spirochaeta pallida* is the causative organism and therefore
the true antigen, this organism does not give good test results as an antigen
and is not so used. In its place, alcoholic extracts of lipids * from animal
tissue are used as antigens. The ultimate understanding of the relationship
between lipid tissue extracts and syphilis, as well as of other fundamental
problems in this field, will come, most likely, not alone from immunologic
investigations, but largely from those of chemists and more especially colloid
chemists.

Interest in the laboratory diagnosis of syphilis may be said to begin with
Schaudin and Hoffman's discovery of the *spirochaeta pallida* in 1905. One
year later, Bordet and Gengou rounded out their studies on the immunologic
phenomenon of complement fixation, which will be discussed later. In the
application of this phenomenon to syphilis by Wassermann, Neisser and Bruck,
the Wassermann test was discovered. This test was believed by Wassermann
to be a specific antigen-antibody reaction, the *spirochaetae pallidae* or their
end products acting as the antigen, and the immune substances of syphilitic
serum as the antibodies. As is well known, Wassermann originally employed
as an antigen an aqueous extract of syphilitic liver which contained large num-
bers of spirochetes and their end products. There seemed therefore no reason
to doubt that the Wassermann test was a specific immunologic reaction.

Soon Wassermann's view of the specificity of his test began to be ques-
tioned. Landsteiner in Germany and Levaditi in France showed that aqueous
or alcoholic extracts of non-syphilitic liver could be used as antigens in the
Wassermann test. Indeed, extracts of other tissues were also found to give

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* This term is used, instead of lipoids, in accordance with the recommendation of the International
Congress of Pure and Applied Chemistry.

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good results; it was further found that superior antigens could be obtained by using alcoholic extracts of normal heart muscle. These last extracts began to be used as antigens in the Wassermann test, and to this day they are used in this test, as well as in the different precipitation tests for syphilis.

Turning to the technical nature of the Wassermann test, it may be said to embody two phases, the first consisting of the interreaction between serum and antigen, and the second, the indicator phase, consisting of a "hemolytic system" which makes this interreaction visible. The technical complexities of the Wassermann test and the sources of error which are inherent in it, led workers to attempt to devise a simpler serum test for syphilis. As early as 1907 Michaelis attempted to develop such a test. This worker employed an aqueous extract of syphilitic liver as an antigen and syphilitic serum as the antibody, observing as a result, the formation of a precipitate. A year later, Jacobstahl observed that on mixing syphilitic serum with a diluted alcoholic liver extract, one could observe the formation of floccules by means of the ultramicroscope. These attempts were followed by precipitation methods by Bruck and Hidaka, Hecht, Meinicke, Sachs and Georgi, Vernes, Dryer and Ward—the last four gaining some renown—and a number of others.

It is obviously difficult in a chapter such as this to present an extensive discussion of the various precipitation tests proposed for syphilis. The aim of this chapter will be to discuss the author's observations on the general phenomenon of precipitation in syphilis and to present the Kahn reaction. This will be followed by a discussion of the phenomenon of complement fixation and of the Wassermann reaction. Although, historically the Wassermann precedes the Kahn reaction, it seemed best, for the sake of simplicity in presentation, to discuss the latter reaction first.

II. The Phenomenon of Precipitation

Although a large number of precipitation tests have been proposed for syphilis, comparatively little has been published on the phenomenon of precipitation. The reason for this may be that it was assumed by different authors that the precipitation phenomenon in syphilis was basically identical with the precipitation phenomenon in immunity. (Kraus, 1897.) However, in the latter phenomenon, specific antigen is employed with its homologous antiserum, and the resulting precipitate consists largely of serum globulins; while in syphilis, an alcoholic heart-extract antigen is employed with syphilitic serum and the precipitate consists largely of lipids. Indeed, our studies indicate that the precipitation phenomenon in syphilis is governed by factors which do not to the same degree govern the precipitation phenomenon in immunity, and in this chapter the former will be presented independently of the immunity phenomenon.

The first observation of the writer in connection with the phenomenon of precipitation in syphilis, was that concentration of the ingredients which enter into a precipitation reaction tended to hasten precipitation, while dilution of the ingredients tended to delay or prevent precipitation. This suggested the use of a concentrated antigen and undiluted serum as the basis for a practical test. Later, it was shown that excessive concentration of the antigen will also delay or prevent precipitation and that an optimum state between concentration and dilution of antigen was necessary for ideal results. The second observation was that the lipid antigen could be so mixed with physio-

logic salt solution, as to produce a suspension that would give immediate precipitation reactions with syphilitic serums. Then came to light important quantitative relationships between the amounts of antigen suspension and serum, also the fact that agitation of the ingredients hastened the formation of precipitates.

These observations led to the evolvement of a highly practical precipitation method for syphilis. This method will be presented later, our aim at present being to discuss briefly the ingredients which enter into the precipitation reaction and the factors which govern this reaction.

Ingredients of the Precipitation Reaction

The ingredients which enter into the precipitation reaction are (1) serum, (2) antigen and (3) physiologic salt solution. These will be discussed with special reference to the Kahn test.

(1) *Serum.* In immunity precipitation reactions, the blood serum from immunized animals contains antibodies in the form of precipitins. Whether syphilitic serum contains similar precipitins is not yet determined. As is true with immune precipitins, the corresponding substances in syphilitic serum (as well as in spinal fluid), are associated with the globulin fraction. It is claimed, however, by some (Forssmann) that the active agent in syphilitic serum is not a globulin, but a lipid associated with the globulin. The fact that the precipitate resulting from the interreaction of immune serum and specific antigen consists largely of globulin, while the resulting precipitate in syphilitic serum consists largely of lipids, would indicate some difference between the two types of reactions.

When mixing syphilitic serum with antigen, the precipitation results vary depending on whether or not the serum has been previously heated. The usual heating method consists in placing the serum in a water bath at 56° C. for thirty minutes. Serums thus heated give more marked precipitation reactions than unheated serums. When extending the heating period to one or two hours, the precipitation results become still more marked. Some workers employ unheated serum in different precipitation tests. But these tests require extended incubation at 37° C. before reading the final results. Prolonged incubation at 37° C. may have a similar effect on the serum as a short heating period at 56° C.

It is possible that heating reduces the stability of the serum globulins of syphilitic serum, facilitating thereby their union with the antigen. Heating, however, will not induce precipitation (with antigen) in the case of non-syphilitic serum. The effect of heating on syphilitic serum may be to decrease the action of protective colloids. In this connection it is of interest to note that the addition of acid or alkali to serum, affects the reaction to an even more marked degree than does heating of the serum at 56° C. If a non-syphilitic serum is excessively acidified—to an acid concentration of 0.03 *N* HCl—it will produce positive precipitation reactions when mixed with antigen suspension. Similarly, if alkali is added to syphilitic serum to a concentration of 0.025 *N* NaOH, there will result negative precipitation reactions with antigen suspension. Lesser amounts of acid or alkali appear to have comparatively little effect on the serum, due undoubtedly to the buffer action of the serum constituents.

(2) *Antigen.* Of the ingredients in the lipid antigen which play a rôle in the reaction with syphilitic serum, only two are definitely known, namely,

crude lecithin and cholesterol. Purified lecithin, according to MacLean, lacks antigenic properties. Cholesterol by itself also lacks such properties. So far as alcoholic extracts of heart muscle are concerned, all appear to have some antigenic powers, no matter how they are prepared, and such extracts when mixed, under proper conditions, with syphilitic serum, will produce precipitates after sufficient incubation. An antigen which is to give desirable results in a practical precipitation test must, however, conform to a number of requirements. When employed with serum, the antigen must possess marked specificity and sensitiveness. It must not be so highly potent as to give occasional reactions with non-syphilitic serums, or so insufficiently potent as to lack sensitiveness. After experimental studies with different antigens used in the Wassermann test, one recommended by Neumann and Geiger for this test seemed especially promising, and as it is now prepared the antigen conforms with the above requirements to a marked degree. Briefly, the antigen consists of coordinated amounts of ether and alcohol extractives of powdered beef heart combined with 0.6 per cent cholesterol. Thus 25 grams of powdered beef heart are extracted at room temperature with 100, 75, 75 and 75 cc. ether, successively—at 10-minute intervals. This removes the fat and the larger amount of ether soluble lipids, leaving an amount of such lipids unextracted which is sufficient for antigen sensitiveness. The beef heart is now extracted with alcohol for 3 days at room temperature, after which the cholesterol is added to the alcoholic extract.

After preparation, each lot of antigen is standardized to the desired degree of sensitiveness with syphilitic serum. Standardization is important because we are obliged to resort to heart muscle for antigen preparation. Due to the variable animal diets and to the extent of autodigestion of the heart muscle after slaughter, and possibly to other causes, different hearts occasionally produce antigens of different sensitiveness. The method for overcoming this difference in sensitiveness is based on the following experimental observations: (1) The extent of the preliminary ether extraction of the beef heart plays an important rôle in determining antigen sensitiveness. Thus, excessive extraction with ether tends to reduce sensitiveness, while meager extraction, up to certain limitations, tends to increase antigen sensitiveness. (2) The extent of alcoholic extraction of the beef heart also affects antigen sensitiveness—excessive as well as insufficient concentration of lipids tending to reduce sensitiveness. It is comparatively simple to apply these observations in correcting antigens to standard requirements. 1. If an antigen is insufficiently sensitive due to meager lipid content (which can be readily recognized by the water-clear instead of somewhat milky and opalescent appearance of the mixture of antigen suspension and salt solution), it is corrected by addition of ether or alcohol extractives from ground heart muscle. 2. If an antigen is insufficiently sensitive due to excessive lipid concentration, it is diluted with alcohol containing 0.6 per cent cholesterol. 3. If an antigen is more sensitive than standard requirements, it is similarly diluted.

In all cases of antigen correction, the extent of dilution or concentration of an antigen is determined by trial tests with syphilitic and non-syphilitic serum, employing standard antigen as a control.

(3) *Physiologic Salt Solution.* This is the usual 0.9 per cent sodium chloride solution, commonly referred to as normal saline. When this solution is mixed under proper conditions with antigen, a lipid suspension is obtained which gives immediate precipitation reactions with syphilitic serum. Any in-

crease in the salt concentration of the solution, increases the sensitiveness of the precipitation reactions, until about 3 per cent salt concentration is reached when non-syphilitic serums begin to give weak reactions. Decreasing the salt concentration below 0.7 per cent, produces weaker reactions, while extremely weak precipitation is obtained if distilled water is employed instead of salt solution. The rôle of the sodium chloride in hastening precipitation may be similar to the influence of electrolytes in other colloidal precipitation reactions.

OPTIMUM CONCENTRATION OF ANTIGEN AND OF SERUM

The importance of optimum concentration of antigen in the precipitation reaction for syphilis, has already been referred to. The following experiment illustrates the extent to which excessive concentration, as well as excessive dilution, is inhibitory to precipitation. A portion of standard antigen is concentrated and another is diluted 50 per cent. The concentration is accomplished by evaporating, let us say, 10 cc. non-cholesterolized antigen and dissolving the residue in 20 cc. cholesterolized antigen. In the case of the dilution, to a given amount, such as 20 cc. standard antigen, are added 20 cc. alcohol containing 0.6 per cent cholesterol. In both cases, the concentration of cholesterol is thus uniform. The modified antigens are mixed with salt solution according to their respective titers *—employing, in each case, the minimum amount of salt solution which will produce a readily dispersable lipid suspension. When these suspensions are employed with syphilitic serum, however, the precipitation reactions are much weaker than the reaction obtained with the unmodified antigen, indicating that excessive concentration as well as excessive dilution of antigen interferes with precipitation.

It is well to point out in this connection that excessive dilution of the tissue-extract lipids in a given antigen, may render the final antigen suspension unfit for use with serum, due to the separation of cholesterol crystals. Thus, it is known that on mixing an alcoholic solution of cholesterol with salt solution, the separation of the cholesterol takes place immediately. The same holds true if instead of an alcoholic solution of cholesterol, a cholesterolized antigen, weak in extract lipids, is employed. The presence of some minimum amount of heart-tissue lipids is necessary to prevent the separation of cholesterol from an antigen suspension. The cholesterol apparently combines with the other tissue lipids in the antigen, with the result that no free cholesterol is under ordinary conditions observed in the antigen suspension.

Turning to serum, no attempt was made to study the effect on the precipitation reaction of concentrating the precipitating substances. A serum rich in such substances reacts best with a larger rather than a smaller amount of antigen suspension, and when the amount of suspension is reduced to about one-twentieth or less of the serum amount, the precipitation reaction may be negative. When diluting such a serum 1 : 5 or 1 : 10 with salt solution and then employing one part of the diluted serum with one-twentieth of antigen suspension, marked precipitation reactions are usually obtained. As in the case of the antigen suspension, an excessive number of serum substances in relation to antigenic substances are apparently inhibitory to precipitation. The same holds true when the serum is excessively diluted and the number of reacting substances are greatly reduced.

* See definition of titer given later.

Quantitative Relationship between Antigen and Salt Solution

On mixing alcoholic-extract antigen with physiologic salt solution, there results either a suspension of lipid particles or what appears to be an opalescent mixture. In the case of the Wassermann test, Sachs and Rondoni first showed that if the salt solution is added to the antigen drop by drop, the final product is turbid due to finely suspended lipid particles; they reported that this product is more sensitive with serum than if the salt solution is added rapidly to the antigen, when the final product is opalescent and free from turbidity. In the case of the author's test, it was found that when the salt solution is added rapidly to the antigen previously described, the resulting suspension is turbid and consists of relatively large lipid particles. The outstanding feature of these particles is that they are immediately dispersed when brought in contact with additional salt solution or serum. Furthermore, in the case of syphilitic serum, a new precipitate appears within a few seconds or a minute or two, while in non-syphilitic serum no precipitate appears.

Immediate precipitation reactions with syphilitic serums will, however, result only when optimum antigen-saline suspension is used. In order to understand how this optimum suspension is obtained, it will be well to look into the quantitative relationship between antigen and salt solution. If a given amount of the author's antigen, such as 1 cc., is mixed with varying amounts of physiologic salt solution, beginning, let us say, with 0.05 cc. and extending to 3 cc., a definite picture is obtained. When 0.05 cc. salt solution is added to the 1 cc. of antigen, no separation of lipids takes place except for a few cholesterol crystals. The antigen remains clear also after the addition of 0.1 cc. or 0.2 cc. of salt solution. When the amount of saline added to 1 cc. antigen is increased beyond 0.2 cc., the final mixture appears turbid, due to the presence of a fine suspension of lipid particles. On further increasing the salt solution amount by 0.2 or 0.3 cc., the suspended particles assume a larger form and remain so until about 1.5 cc. salt solution are added to 1 cc. of antigen, when the suspended particles begin to be fine again. When 2.5 cc. are added to 1 cc. antigen, the final product is opalescent and free from turbidity. With additional salt solution, the final product shows still greater clarity. We thus have here a condition where an excessive amount of antigen in relation to salt solution as well as an excessive amount of the latter in relation to antigen result in clear solutions, with the formation of precipitates in between the two zones.

Although the general appearance of the lipid particles in the antigen suspension is relatively the same throughout the precipitation zone, their dispersion in salt solution varies markedly. Again taking 1 cc. of antigen as our constant, it will usually be found that with amounts of salt solution less than 1 cc., the lipid particles in the resulting antigen suspension are stable and not dispersed in additional salt solution or serum, whereas if the salt solution is increased beyond 1 cc. the particles in the suspension are unstable and readily dispersed. The proportion of salt solution to antigen at the critical region where the lipid particles change from a state of stability to one of instability, varies with the heart muscle from which antigen was prepared. For any given antigen, the salt solution-antigen ratio just beyond this critical region, where the particles are readily dispersable, is the end point or "titer" for any given antigen. *This titer is defined as the minimum amount of salt solution added*

to 1 cc. antigen, resulting in a suspension of lipid particles which are readily dispersed in the presence of serum or additional salt solution.

Practically all antigens give most sensitive precipitation reactions with serum when mixed with salt solution in the proportion indicated by the titer. Thus, if the titer of a given antigen is 1 cc. antigen + 1 cc. salt solution, then suspensions resulting from 1 cc. antigen + 1.1 or 1.2, etc., salt solution will usually be proportionally less sensitive. Unfortunately different antigens vary quantitatively in sensitiveness even at this titer, due to the variable nature of heart muscle. To overcome these differences in sensitiveness, special methods of correction have been devised. These were briefly discussed under "Antigen." It is needless to add that an antigen-saline suspension containing stable lipid particles, can not be used in practice, since these particles would give the impression of positive reactions with all serums.

The extreme sensitiveness of the antigen-saline suspension in giving immediate precipitation reactions with syphilitic serum, is at least in part explained by the apparent instability of the suspended particles. It might be added that these particles play the exclusive rôle in precipitation with syphilitic serum. Experiments showed that when the particles were separated from the suspended medium by centrifugation and resuspended in a new medium containing an alcohol-saline combination similar in volume to that of the original antigen and salt solution, the suspension in the new medium was as sensitive with syphilitic serum as in the original medium.

Quantitative Relationship between Antigen Suspension and Serum

From the very beginning, our studies indicated that for optimum precipitation results, it is essential that the number of antigenic reacting units should approximate the number of serum units. If the number of antigenic units are excessive, precipitation is inhibited. This can be readily established by employing definite amounts of serum of different potency, with increasing amounts of antigen suspension. The strongly potent serums will produce precipitation reactions with equal amounts of antigen suspension; the moderately potent serums will be negative in these proportions but positive in those where the amount of suspension is about one-third of that of serum. In the case of weak serums, positive reactions will be obtained only in those proportions of serum and antigen suspension in which the latter is reduced to one-twelfth or less than the serum amount. When the amount of suspension is excessively reduced in relation to serum, the bulk of the precipitate is correspondingly reduced, since the precipitate is the end product of the union of serum and suspension.

The effect of an excess of serum units over antigen units, can not be as readily determined as the effect of excessive antigen units. Antigen is a standardized product, whereas in the case of serum each varies from the other in potency. The less potent the serum, the more marked is the precipitation with small amounts of antigen suspension, while the more potent the serum, the more marked is the precipitation with larger amounts of antigen suspension. With highly potent serum it is not uncommon to obtain weak or negative reactions on markedly reducing the amount of antigen suspension. We thus have here a zone phenomenon similar to that frequently observed in other precipitation reactions of colloidal character where an excessive amount of either one or the other reacting substance interferes with precipitation.[1]

* See paper by A. Lottermoser in Vol I of this series *J. A.*

The quantitative relationship between serum and antigen suspension may be summarized as follows: With a constant amount of a given serum, minute amounts of antigen suspension will yield no visible precipitate, some minimum amount of suspension being essential for precipitation. This minimum is dependent on the number of reacting substances in the serum, being greater the more potent the serum. As the amount of antigen suspension is further increased, the quantity of the precipitate is proportionally increased, until a maximum amount of suspension is reached. This maximum also depends on the number of serum substances present, being greater the more potent the serum. When the amount of suspension exceeds the maximum, precipitation is inhibited.

Shaking of Antigen Suspension and Serum Mixture

During our early studies of the precipitation phenomenon in syphilis, we observed that precipitation results varied in the hands of different workers in spite of the fact that the technical details of the procedure were identical. In searching for the cause of this variation in the results, it was observed that agitation of the mixture of antigen suspension and serum markedly hastens the formation of precipitates. Strongly potent syphilitic serums require agitation for but a few seconds, but the weaker serums require several minutes' agitation before showing precipitates. The optimum shaking speed was found to be 275 oscillations per minute. This optimum applies to moderately and weakly potent serums, and not to strongly potent serums. The latter show somewhat heavier precipitation particles at a lower speed. When the precipitation speed extends beyond 350 oscillations per minute, there is a tendency towards the breaking up of the particles into a finer state, thus rendering their reading somewhat difficult. In the case of the weakly potent serums, the longer they are shaken with the antigen suspension, the stronger will be the precipitation reactions. In most cases, maximum precipitation is obtained after on or two minutes' shaking, although in isolated cases, there is a tendency for stronger reactions to accompany an increase in the time of shaking up to five to seven minutes. In the author's test, a three-minute-shaking period is employed as a standard, this length of time being ample for most serums.

Shaking, even when extended beyond ten minutes, does not bring forth precipitates in mixtures of non-syphilitic serum and antigen suspension. Neither does two to three days' incubation of such mixtures bring forth precipitates. It would appear from this that there is a qualitative rather than a quantitative difference between syphilitic and non-syphilitic serum, otherwise one would obtain precipitates in the latter after prolonged shaking or incubation. Shaking hastens the formation of precipitates most likely by mechanically bringing together the serum-antigen substances. It is possible that shaking also reduces the stability of the serum-suspension mixture which factor would also tend to hasten precipitation.

III. The Kahn Reaction

Having shown that it was possible to prepare an antigen and so mix it with salt solution as to produce a suspension which will give rapid precipitation reactions with serum, and having observed several optimum conditions which affect these reactions, the evolvement of a practical precipitation test for syphilis was a natural consequence. Actually, a number of procedures have

been evolved, each capable of giving clinicians special information regarding the serologic condition of the patient. The "routine" or diagnostic procedure corresponds in specificity and sensitiveness to a reliable Wassermann test and will be presented in some detail, while the other procedures will be summarized briefly.

The routine or diagnostic test with serum is a three-tube method involving the use of three different proportions of serum and antigen suspension. The amounts of the latter are 0.05, 0.025 and 0.0125 cc., respectively, while the serum amounts are 0.15 cc. throughout. One occasionally finds a syphilitic serum of such marked potency that it will give a precipitation reaction with an equal amount of suspension. With most serums such an amount is inhibitory to precipitation, and not desirable for a practical test. A three-to-one proportion of serum and suspension was found to be more desirable for "picking up" strongly potent serums. A six-to-one proportion of serum to suspension was chosen in turn to detect moderately potent serums and a twelve-to-one proportion, weakly potent serums. This last proportion is the most sensitive of the three, while the first proportion is the least sensitive. The result is that a serum which is sufficiently potent to produce a precipitation reaction in the first tube, produces such reactions also in the remaining two tubes. A strongly positive $(++++)$ reaction therefore is based on the presence of precipitates in each of three tubes. The moderately potent serums usually show precipitates in the last two tubes, and the weak serums only in the last tube. When the proportion of suspension to serum is less than one to one-twelfth, the precipitation reactions are more sensitive than those taking place in the third tube of the routine test. But it seemed best, especially for reasons of conservatism, to stop with the one-twelfth proportion.

PERFORMANCE OF TEST

The antigen suspension is prepared by mixing this reagent with salt solution according to the required titer. Thus, if the titer is 1 cc. antigen $+$ 1 cc. physiologic salt solution, the following procedure is employed. One cc. salt solution is deposited in a tube of about 1.5 cm. diameter and 5.5 cm. height. One cc. antigen is deposited in a similar tube. The salt solution is then poured into the antigen tube and, without waiting to drain the tube, is poured back and forth five or six times to assure proper mixing. The resulting lipid suspension is ready to be used with serum 10 minutes after mixing, since immediately after mixing the suspension is slightly less sensitive with serum than after a few minutes' standing.

The serum is prepared in the usual manner by centrifuging the blood specimen to throw down the blood clot and cells. The clear serum is heated in a water bath at 56° C. for 30 minutes, and is then ready to be used with the suspension.

After the antigen suspension has stood for 10 minutes, it is thoroughly mixed, and 0.05, 0.025 and 0.0125 cc. quantities measured into the bottom of three tubes. In performing 10 tests, it is recommended to employ the "standard rack" which has three rows for 10 tubes each. The 0.05 cc. quantity is then measured into all the tubes of the first row; the 0.025 cc. quantity into the tubes of the second row and the 0.0125 cc. into the tubes of the third row. The serums are added in 0.15 cc. amounts to each of the 0.05, 0.025 and 0.0125 cc. quantities of the antigen suspension. The rack is shaken for three minutes, the number of oscillations approximating 275 per minute. Then,

in order to simplify the reading of the results, 1 cc. physiologic salt solution is added to the first row of tubes—those which contain the 0.05 cc. amounts of antigen suspension—and 0.5 cc. salt solution to all remaining tubes. The non-syphilitic serums appear opalescent and clear, while the syphilitic serums show the presence of precipitates.

When the precipitates are well marked and suspended in a clear medium, they are read "four plus." When less marked and especially when the medium appears cloudy, indicating incomplete precipitation, they are read three, two, one or doubtful. The final result reported to physicians is the average of the findings of the three tubes. Thus, complete or four plus precipitation in each of the three tubes is twelve plus divided by three, in other words, four plus. If the reading in the three tubes is respectively negative, two plus, and four plus, then six plus is divided by three and the final result is two plus.

The Kahn reaction appears to be specific only for syphilis and yaws, and is apparently not influenced by pathologic conditions other than these. The Michigan State Health Department has reported, with satisfactory results, over 150,000 Kahn tests, without Wassermanns, from October 15, 1925 (when the Kahn test was made the standard method for the serum diagnosis of syphilis), to October 15, 1927. The test was made standard in the U. S. Navy, December, 1925. Since then it has been made standard by the State Health Department of Illinois, West Virginia and by numerous other institutions. The major value of the Kahn test, aside from its marked specificity and sensitiveness, lies in its relative simplicity of technique, its rapidity, its low cost, its relative freedom from sources of error and its availability throughout the world, whether in the tropics, the field laboratory of the Army or aboard ship of the Navy.

Of the additional procedures of the Kahn reaction, the quantitative method deserves mention. This determines the relative number of syphilitic reacting substances in the blood serum, and consists of a series of eight serum dilutions with salt solution ranging from $1:1$ to $1:60$. These dilutions in 0.15 cc. amounts are tested with 0.01 cc. of standard antigen suspension. The tests are shaken three minutes, 0.5 cc. salt solution added to each tube and results read. The reactions in the individual tubes are interpreted as positive or negative, depending on the presence or absence of a definite precipitate. The final result is expressed in syphilitic reacting units according to the formula $S = 4D$, where $S =$ serum potency in terms of reacting units and $D =$ maximum dilution of serum giving a positive reaction. Clinically, this procedure is of especial value whenever it is desired to study quantitatively the serologic effect of anti-syphilitic therapy.

The spinal fluid procedure requires precipitating the globulins in spinal fluid with saturated ammonium sulfate solution, producing 40 per cent saturation, separating the globulins by centrifugation and redissolving them in a quantity of normal saline, one-tenth of the original spinal fluid amount. The redissolved globulin, in a 0.15 cc. amount, is now added to 0.01 cc. of antigen suspension and the test completed after 3 minutes' shaking. The results are interpreted on a plus sign basis. Clinically, the test is of value in the diagnosis and treatment of neuro-syphilis.

Additional procedures of the Kahn reaction include a quantitative spinal-fluid method which gives the relative number of syphilitic substances in this fluid; a presumptive procedure with serum which is more sensitive than the routine diagnostic test; micro-procedures for serum and spinal fluid when

sufficient amounts are not available for the regular tests, and a procedure for fluid from syphilitic lesions.

An outline of the routine or diagnostic Kahn test with interpretation of the results are presented in Table 1.

TABLE I. *Outline of Kahn Test and Interpretation of Results.*

The Test.

Tube No.	1	2	3	Completion of Test
Serum : Antigen suspension	3 : 1	6 : 1	12 : 1	Tubes shaken three minutes, 1 cc.
Antigen suspension,† cc............	0.05	0.025	0.0125	salt solution added to first tube
Serum ‡ (heated at 56° C. for 30 min.) cc.	0.15	0.15	0.15	and 0.5 cc. to other two tubes and results read.

Interpretation of Results.

Reaction No.				Final Result (Average of Reactions of Three Tubes)
1.....................	++++*	++++	++++	++++
2.....................	+++	++++	++++	
3.....................	++	++++	++++	+++
4.....................	+	+++	++++	
5.....................	±	+++	++++	++
6.....................	—	++	++++	
7.....................	—	±	++++	+
8.....................	—	—	+++	
9.....................	—	±	++	±
10...	—	—	++	
11.....................	—	—	+	—
12............	—	—	—	

Some Typical Reactions

† Control consists in establishing that suspension is readily dispersed in salt solution.
‡ Control consists in proving that serum is entirely free from foreign particles.
* ++++ = marked precipitation reaction; +++, ++, + and ± = proportionally **weaker** precipitation.

The colloidal gold reaction of Lange is a precipitation method for syphilitic spinal fluid. In this reaction 5 cc. of a special colloidal gold solution are added to a series of ten tubes which contain 1 cc. of varying dilutions of spinal fluid in 0.4 per cent sodium chloride. It is generally recognized that the globulins present in the spinal fluid are responsible for the changes observed in this reaction, which is a gradual precipitation of the colloidal gold. The results are read after 24 hours, precipitation being recognized by changes in the color of the colloidal gold solution. The degree of aggregation of the particles is indicated by a series of colors, to each of which is assigned an arbitrary number as follows: Cherry red 0, bluish red 1, reddish blue 2, deep blue 3, gray blue 4, colorless 5.

The results are expressed in curves with the tube numbers as abscissae and

the color or aggregation numbers as ordinates. With a negative spinal fluid no color change occurs; the gold solution remains cherry red, and the curve is represented by a flat baseline. Three curves are met with that have been found to have clinical significance. At the extreme left is the paretic zone where the change usually reaches as high as 5 and gradually passes down to the base line somewhere beyond the middle of the graph. Such a curve is highly significant of dementia paralytica. The middle zone is known as the luetic zone. Here the change usually starts at the base line, rises as high as 3 and then returns to the base line. This type of curve is given by patients with neurosyphilis of the non-parenchymatous type but it is not exclusively diagnostic of such a condition, since a curve of similar character is obtained in other conditions. The zone at the right end of the series is known as the meningitic zone; spinal fluids giving changes in this zone indicate suppurative or tuberculous meningitis.

The colloidal gold solution may be prepared * by adding 10 cc. of a 1 per cent solution of c. p. gold chloride to one liter of doubly distilled water, heating to 60° C., and then adding 7 cc. of a 2 per cent solution of potassium carbonate solution; upon continued heating to 80° C., ten drops of a 1 per cent solution of oxalic acid solution are added, and finally at 90° C. 5 cc. of a 1 per cent solution of neutral formaldehyde solution is added. The solution is then shaken until the characteristic changes in color from blue through lilac to bright cherry red takes place. The solution must be neutral; it should be clear to transmitted light but show a slight shimmer with reflected light and should give a positive and negative curve when set up with known spinal fluids.

Tests similar in principle to the colloidal gold test but which have as their base opalescent colloidal solutions of gums are also employed. Among these may be mentioned the gum mastic test and the colloidal benzoin test. The tests are set up similarly to the colloidal gold test, numbers being assigned to the varying degrees of precipitation of the opalescent solutions, and precipitation curves drawn. These tests are not so satisfactory as the colloidal gold test, since the differences between positive and negative reactions are not as sharply defined as are those in the colloidal gold reactions.

IV. The Phenomenon of Complement Fixation

In order to understand the phenomenon of complement fixation, it will be necessary to bring to mind the antigen-antibody reaction which results in lysis or dissolution of the antigenic substance. If some foreign cellular elements such as red corpuscles are injected into an animal, there will appear in its blood hemolytic antibodies which will be capable of rupturing the corpuscular wall and dispersing the cell content. These antibodies are spoken of as hemolysins, and differ ·from precipitins and agglutinins in that they need an additional "complementary" substance before they can bring about hemolysis. Complement may be defined as a non-specific immune substance and is present in freshly drawn blood. An essential difference between complement and hemolysin is that the former is thermolabile, while the latter is thermostable.

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* Precise details for making colloidal gold for clinical use are given, e.g., by O. I. Lee in a paper entitled "A simple procedure for preparation of colloidal gold for diagnostic purposes," in *Am Journal of the Medical Sciences*, 155, p. 404 (March, 1918). Here ordinary distilled water is used. J. A.

It will be recalled that complement does not play a rôle in precipitation (and agglutination) reactions, such reactions depending only on specific antigen-antibody complexes. These complexes have, however, a marked affinity for complement and will readily remove it from solution, probably by adsorption. This removal of complement by antigen-antibody complexes, is known as "complement fixation." Only specific or homologous antigen-antibody complexes are capable of fixing complement; antigen alone or immune serum alone or heterologous antigen-antibody mixtures can not fix complement, excepting insignificant traces.

In practice, the complement fixation reaction is utilized in determining whether or not a given serum-antigen mixture is homologous. Suppose it is desired to establish whether an unknown serum contains antibodies specific with regard to a known antigen. The first step is to destroy the native complement of the serum by heating it in a water bath, usually for 30 minutes at 56° C. Fresh complement, added to a mixture of the serum and antigen, will then be fixed if the serum is homologous with the antigen, and remain free if the serum is heterologous. Since the fixation of complement is an invisible phenomenon, the rôle of complement in hemolysis is now utilized for its detection. With this end in view hemolysin and corpuscles are added to the serum-antigen-complement mixture. Should hemolysis of the corpuscles occur, complement obviously was not fixed, proving that the unknown serum did not contain specific antibodies. If hemolysis does not occur, complement must have been fixed by a specific antigen-antibody complex, and our unknown serum therefore contained specific antibodies.

The determination by complement fixation of whether a given serum comes from a syphilitic or non-syphilitic patient, is made in a similar manner, except that the antigen employed consists of an alcoholic extract of heart tissue and is not specific in an immunologic sense. This determination is in practice referred to as the Wassermann test for syphilis. The ingredients of this test are tissue-extract antigen, physiologic salt solution, serum and the hemolytic system consisting of complement, hemolysin and red blood cells. Since the first three ingredients have been fully discussed in presenting the precipitation reaction, only the hemolytic system will be considered here.

1. *Complement, Hemolysin and Red Corpuscles.* Fresh guinea-pig serum is usually employed as complement, because experience has shown that this animal's serum almost invariably possesses marked hemolytic powers. As already indicated, complement is present in normal serums both in immune and non-immune animals. The origin of complement is not definitely established. Some claim that the leucocytes are the source of this ingredient. An interesting property of complement is that it is readily fixed or destroyed by various agents, such as kaolin, agar and a number of chemical substances. Both acid and alkali inhibit its action. It is thermolabile, being destroyed by heating at 56° C. for 30 minutes or less. According to Ehrlich, complement consists of at least two groups—one (the haptophore), by which it unites with the antibody, and the other (the toxophore), which unites with the antigen.

Hemolysin (amboceptor) is obtained by immunizing rabbits with red corpuscles. Usually sheep corpuscles are employed because of the relative simplicity in obtaining blood from this animal. The blood is drawn into a sterile glass vessel containing beads, and shaken for several minutes to remove the fibrin and thus prevent its clotting. The corpuscles are then centrifuged, the serum removed and the cells washed several times with physiologic salt

solution to remove remaining traces of serum. A given quantity of washed cells, such as 1 or 2 cc., are injected into rabbits, either intravenously or intraperitoneally. The injections are repeated three or four times and about a week after the last injection, it will be found that the serum of the rabbit contains some hemolysin which will dissolve sheep corpuscles in the presence of complement.

The red corpuscles are obtained usually from the jugular vein of the sheep. The serum is separated by centrifugation and the corpuscles washed with salt solution to remove traces of serum. The corpuscles are then suspended in salt solution, making a suspension of 2 to 5 per cent, the concentration depending on the complement fixation method employed.

Complement, hemolysin and the corpuscles must be properly standardized both individually and with reference to the other reagents of the Wassermann test. In the procedure of this test to be here presented, the corpuscle suspension consists of 5 per cent washed and packed cells suspended in physiologic salt solution, and 0.1 cc. of this suspension is employed under all conditions as the standard amount. The unit of hemolysin is the smallest amount which will hemolyze 0.1 cc. of the standard suspension of corpuscles in the presence of an arbitrary amount of complement after 15 minutes' incubation in the water bath at 37° C. This complement amount is 0.1 cc. of a 1 : 10 dilution of fresh guinea-pig serum with salt solution, chosen empirically because experience has shown that this amount contains approximately "2 units" of this ingredient. Usually, the serums from several guinea-pigs are pooled for this purpose. After determining the hemolysin unit, it is employed in establishing the complement unit. This unit is the minimum amount of complement which will hemolyze 0.1 cc. of the sheep cell suspension in the presence of 0.1 cc. (2 units) of hemolysin after 15 minutes' incubation in the water bath. It should be pointed out that practically every Wassermann technique has a different unit system; not only do the amounts of complement, hemolysin and corpuscles vary, but also the period of incubation employed to bring about hemolysis.

V. THE WASSERMANN REACTION

It has already been indicated that originally Wassermann, Neisser and Bruck employed a watery extract of syphilitic liver as an antigen in the complement fixation test for syphilis, and that they believed the test to be a specific immunity reaction. When alcoholic extracts of animal tissue began to be employed as antigens in the test, there was a tendency to vary greatly their method of preparation. These variations, however, were not limited to antigen preparation; every step of the Wassermann procedure underwent numerous modifications with the result that to this day practically no two workers employ identical methods.

In presenting the technique of the Wassermann test in this chapter, it seemed best, for the sake of simplicity, to employ as the serum-antigen complex the components of the completed Kahn reaction. That the hemolytic system can be added to completed Kahn tests, thereby obtaining complement fixation results, has been experimentally demonstrated in this laboratory. In attempting to study the identity of complement-fixing and precipitin substances in syphilitic serums, the hemolytic system was added to one of the tubes of the completed Kahn test, and the final complement fixation results compared with the original Kahn results. In 1,800 comparative precipitation-comple-

ment fixation tests, we obtained 93 per cent absolute agreement, 5 per cent relative agreement and 2 per cent. disagreement. With greater knowledge of the two reactions, there is reason to believe that still greater agreement might be obtained.

PERFORMANCE OF THE WASSERMANN TEST

The routine diagnostic Kahn test is completed in the usual manner and the results recorded. From the third tube of the test (which contains the least amount of antigen suspension) 0.15 cc. of the serum-suspension mixture is withdrawn and deposited in a small test tube. In two similar tubes are deposited individual amounts of serum and suspension respectively equivalent to the amounts of each present in the above mixture. Two units of complement (0.1 cc.) are now added to each of the three tubes. The mixtures are placed in the water bath at 37° C. for 30 minutes to permit fixation of com-

TABLE II. *Outline of Wassermann Test and Interpretation of Results.*

The Test.

Tube No.	1	2	3
Serum-antigen suspension + saline (from 3rd tube of Kahn test) cc.	0.15	....	....
Antigen-suspension control cc.	....	0.003	....
Serum control cc.	....	...	0.035

Two units (0.1 cc.) complement added to each tube and mixtures placed in water bath at 37° C. for 30 minutes.

Hemolysin (2 units) cc.	0.1	0.1	0.1
Red cell suspension cc.	0.1	0.1	0.1

Mixtures again incubated at 37° C. for 15 minutes.

Interpretation of Results.

Reaction No.	Hemolysis			Final Result
1................	None	Complete	Complete	++++
2................	Slight	Complete	Complete	+++
3................	Moderate	Complete	Complete	++
4................	Considerable	Complete	Complete	+
5................	Complete	Complete	Complete	—
6................	None	None	None	"Anti-complementary"

plement. The hemolysin solution, also in a 0.1 cc. amount containing two units, and a 5 per cent suspension of packed sheep corpuscles are now added to each tube and incubated for about 15 minutes in the water bath at 37° C.

It will be found that the tube which contains serum without suspension as well as the one which contains suspension without serum will show complete

hemolysis of the corpuscles since the complement in these tubes remained free. If the tube containing suspension and serum originally showed a positive Kahn reaction, indicating that the serum came from a syphilitic patient, it will show a positive Wassermann reaction, namely, absence of hemolysis due to the fixation of complement by the syphilitic-serum-suspension complex. If the Kahn tube showed a negative reaction, indicating that the serum came from a non-syphilitic patient, it will show a negative Wassermann reaction, namely, complete hemolysis of the blood cells as in the two control tubes. Gradations of hemolysis are interpreted on a four plus basis as in precipitation reactions. Thus, complete absence of hemolysis is interpreted four plus; degrees of partial hemolysis are indicated by three, two and one plus, while complete hemolysis is read as negative.

It might be added that the complete Kahn test may be utilized with any complement fixation technique. It is well to use the tube of the Kahn test which contains the least amount of antigen suspension, since an excessive amount of suspension by itself may adsorb an appreciable amount of complement. From this tube, an amount of serum-suspension mixture is pipetted off, corresponding to the amount of serum to be employed in the desired complement fixation technique. Complement is then added, the mixture incubated and the complement fixation test completed.

VI. Discussion and Summary

The subject of serum diagnosis of syphilis was presented in this chapter not in conformity with historical aspects, but with our newer knowledge of this field. The observations dealing with the phenomenon of precipitation which were summarized briefly, represent an attempt to place serum reactions in syphilis in the realm of quantitative science. Each of the observations recorded—the necessity for optimum concentration of the ingredients, the relationship between antigen and physiologic salt solution, the production of a markedly unstable antigen suspension, the relationship between this suspension and serum, and the importance of agitation of the ingredients—are the results of quantitative experiments. With the accumulation of facts regarding the phenomenon of precipitation, the evolvement of a practical diagnostic method for syphilis was a natural consequence.

That precipitin and complement fixing substances in syphilitic serum are in all probability identical, was suggested by Jacobstahl as early as 1908. The close agreement between precipitation and complement fixation results obtained by adding the hemolytic system to completed Kahn reactions, corroborated this view. The nature of the inter-reaction between serum and antigen suspension, is unquestionably the same in the Kahn and Wassermann tests. The end result of this inter-reaction being readily visible in the former test, no further steps are necessary; the end result not being visible in the case of the Wassermann test, a special indicator in the form of the hemolytic system is employed to render it visible. With this view in mind, it appeared logical to present the technique of the Wassermann test by combining the serum-antigen complex of the completed Kahn reaction with the hemolytic system. It should be added that the technique of neither the Kahn nor the Wassermann test was presented in full detail. The aim has been rather to give a general picture of the two methods and emphasize especially the phenomena underlying them.

The extent to which the field of serum diagnosis of syphilis offers problems to colloid chemists, was indicated in the beginning of this chapter. The same thought we should like to emphasize in closing. The studies on the phenomenon of precipitation discussed above make a beginning which should be extended until the mechanism of the reaction is fully understood. Furthermore, until the nature of the substances in syphilitic serum which cause precipitation (or complement fixation) is known, these syphilis reactions must remain empirical. The complement fixation reaction, due to its complexity, its dependence upon fresh animal reagents, its titrations and incubations, has not served well as a medium for fundamental research. The Kahn reaction, however, being relatively simple, especially lends itself to research investigations, and there is reason to believe that it will play an important rôle in solving many of the perplexing problems in this field.

Some Physico-Chemical Characteristics of Immune Serum

Serum

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Experiments made during the past four years on immune sera have given us some interesting and precise information concerning their peculiar physico-chemical behavior. It is well known that a normal and an immune serum are totally different from a biological standpoint. This is easily ascertained by the biological tests, hemolysis, precipitation, or agglutination, according to the nature of the antigen used in immunizing the animal.[1] But, unless the immune serum is placed in contact with its specific antigen, there is no means of differentiating it from a normal serum. Chemically, its composition is the same; physically, its properties, density, viscosity, refractive index, conductivity, etc., are unaltered. Yet immune serum is different.

Certain authors have observed[2] that in some cases, after immunization, the percentage of globulins in serum was increased in respect to albumin. P. Lecomte du Nouy and Baker[3] were unable to control this in experimenting with rabbits. It is, therefore, not general, and in any case does not imply any chemical change in the constituents of the serum, nor would it explain the phenomena which we are about to describe.

It is not surprising that the usual methods of chemical analysis did not enable us to detect the difference in immune serum. It is known, indeed, that a very large number of substances—probably all the proteins and a number of other bodies—can act as specific antigen, that is, are capable, by reacting with some constituent of the living organism, of conferring upon it different chemical properties with respect to the same antigen. The differences may protect or sensitize the organism, according to the substance injected.[4] But no matter what they are or how important, the fact remains that the nature of the chemical elements in the constitution of the plasma is unchanged. This clearly shows that our ordinary chemical methods of analysis are unable to respect the integrity of certain complicated and fragile chemical individuals which exist only in the plasma, and which are endowed with specific chemical properties deriving from their existence as a unit. Once this individuality is destroyed, these properties are lost, and the chemist, like the wrecker, finds nothing but the stones from which the monument had been built.

Therefore, it was desirable to find a method whereby all the unknown chemical individuals could be studied without first destroying their individuality. No one can tell as yet how the numerous elements of the serum are bound together. The fact that proteins, lecithin, fats, fatty acids, and cholesterol are found does not carry with it the conclusion that they all exist separately in the serum. We know that this cannot be. As they appear in the serum in solution and as some of them are perfectly insoluble when isolated, it is impossible to state at present that a "molecule of serum" or a "molecule of plasma" does not exist as a unit.

It is quite obvious that the chemical study of such an enormous molecule cannot as yet be attempted. It is possible to conceive that every active group could react individually, thus changing its properties with respect to one single substance without, however, greatly altering the general properties of the whole with respect to other simple reagents. But if only a few atoms are shifted [5] on this mass of, say, 400 atoms or more, or if some new atoms are added to it, there must be a slight change in the field of forces surrounding this molecule. If all these slight changes could be summed up, a relatively important modification would be expected. And if, somehow, an enormous number of molecules could be arranged side by side so that this addition could take place, it might even perhaps be measured.

This is exactly what was done in producing monomolecular layers of serum on water. Something like 130 thousand billions of molecules, if it is assumed that their cross section is about the same as that of the egg albumin molecule, are placed vertically in a sort of geometric mosaic. The most minute change in the field of forces binding these molecules together will be amplified more than one hundred thousand billion times, and the roughest measurement of static surface tension may detect it.

This hypothesis, if true, would lead to the somewhat unexpected conclusion that the phenomenon should be more marked with highly diluted than with pure serum. Consequently, when the watch-glasses previously described were used, it was around the dilution of 1/10,000 that a phenomenon of maximum amplitude would be expected. In order to verify this, the first series of experiments was performed in the following way: [6]

Measurement of Surface Tension. Measurements were made with the du Noüy tensiometer, according to the technique described in former papers,[7] and the time-drop in 2 hours was observed. All measurements were made at 23° C. ± 0.5.

Animals. 31 rabbits, 4 dogs, and 18 chickens were used. 15 to 20 cc. of blood were removed before the antigen was injected, in order to study the normal serum. The same amount was withdrawn 15 days after the injection. In certain cases, however, the animal was bled every day in order to follow the evolution of the phenomenon.

Antigen. Dog serum, white of egg, and red corpuscles of rabbits and sheep were used. For the latter, the blood was received in a sodium citrate

[5] The number of possible combinations in such complex substances is beyond imagination. As an illustration, it may be recalled that twenty people seated around a table, that is in a two-dimensional space, can be placed in 20! different ways, i.e., two billion billion ways. One egg albumin molecule contains more than 4,000 atoms, distributed in a three-dimensional space. Therefore, the number of possible combinations, though it can be calculated, is impossible to conceive, yet each corresponds theoretically to a definite chemical property.

[6] Lecomte du Nouy, P., *J. Exp. Med.*, 37, 659 (1923); Surface Equilibria of Biological and Organic Colloids," New York, 1926.

[7] Lecomte du Nouy, P., "Series on Surface Tension of Serum," I to XV, *J. Exp. Med.*, 35, (1922 to 1927).

anti-coagulating solution and centrifuged. The red corpuscles were then washed three times in NaCl isotonic solution, centrifuged again, and the normal volume was restored by means of the isotonic solution. Usually, four intravenous or intraperitoneal injections of 4, 6, 8, and 10 cc. were made. The white of egg was injected in isotonic solution at 20 per cent.
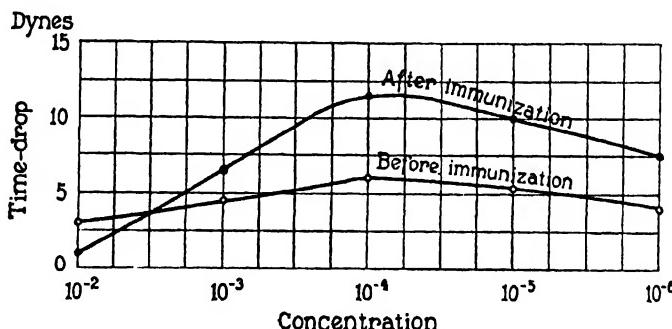
FIG. 1

Controls. Control animals were bled at the same time as the experimental animals and received injections of isotonic salt solutions, homologous red cells, and turpentine (0.3 cc. under the skin). This was done in order to make sure that the phenomena observed were a consequence of the immunization process and not due to the injection of a non-antigenic substance.
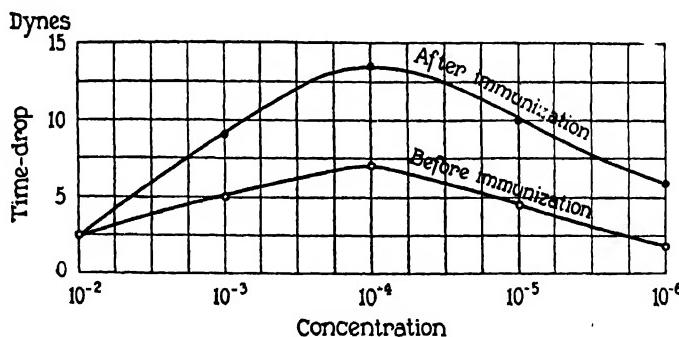
FIG. 2

The state of immunization of the animals was followed, according to the customary techniques, hemolysis or precipitation.

The results obtained showed clearly that the value of the drop of the surface tension in two hours was increased in all the animals which had been submitted to an antigenic injection, while the control animals did not show any change. Moreover, the hypothesis concerning the amplitude of the phenomenon at different concentrations was verified. Text-Figures 1 and 2 show that a maximum exists at 1/10,000, as had been foreseen.

This is probably the first time that the physico-chemical modifications of the serum consecutive to antigen injection have been demonstrated by a direct and purely physical method.

TABLE I. *Value of the Time-Drop of the Serum of Six Rabbits During the Process of Immunization and of Two Controls.*

Date	Controls		Rabbits Injected with Sheep Cells			Rabbits Injected with Egg White			Remarks
	No. 9	No. 10	No. 11	No. 12	No. 13	No. 14	No. 15	No. 16	
1922	Dynes	Dynes	Dynes	Dynes	Dynes	Dynes	Dynes	Dynes	
Sept. 28	4 5	6.0	8.0	7.7	6.2	4.2	5.2	4.5	First injection
" 29	3.0	5.5	6.0	7.0	10.0	8.0	10.0	12.5	
Oct. 2	4.5	2.0	9.0	7.5	10.5	9.5	9.0	6.0	
" 3	4.5	5.0	5.2	7.5	6.5	4.5	7.0	5.0	Second injection
" 4	2.5	2.0	4.5	4.5	4.0	3.5	4.5	7.5	
" 5	1.5	3.0	9.0	10.0	. . .	8.0	4.0	12.0	
" 6	1.8	6.2	9.0	12.5	11.0	12.7	13.0	10.8	Third injection
" 9	5.0	11.0	10.5	13.0	7.5	5.5	6.5	10.5	Fourth injection
" 10	5 5	7.0	11.0	12.0	17.5	11.5	14.0	15.0	
" 11	8.0	7.0	14.5	13.0	11.0	15.5	17.3	17.5	
" 13	5.5	7.0	. . .	14.8	13.5	13.5	14.0	14.0	
" 16	3.0	5.5	Died	11.0	13.5	13.5	12.5	10.5	

The existence of a measurable phenomenon being thus ascertained, the next step was to study its evolution as a function of the time. Table I expresses the results of a series of experiments with six rabbits and two controls. Text-Figures 3 and 4 illustrate two other series, one with six and the other
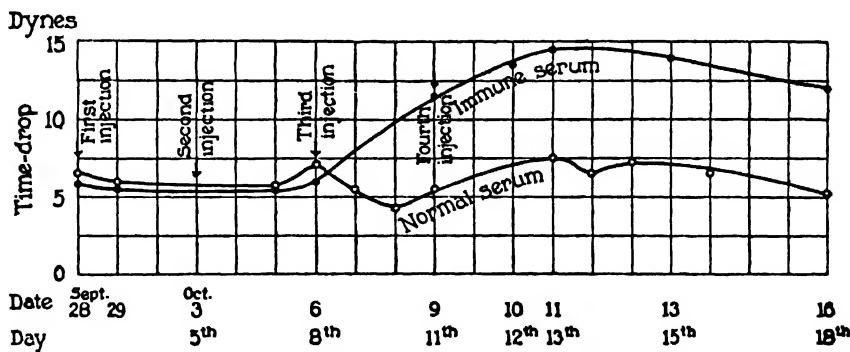
FIG. 3

with eight rabbits. In these last cases, the control curve expresses the mean values obtained with eight animals. These curves are quite analogous to those obtained by plotting in ordinates the quantity of antibody resulting' from similar experiments and titrated according to the ordinary methods *in vitro.*[8] A parallelism seemed, therefore, to exist between the phenomenon

[8] Jorgensen, A. and Madsen, T., "Festskrift. ved. Indvielsen af Statens," Paper 6, Copenhavn, Serum-Institut, p. 12 (1902). Fischer, A., *J. Exp. Med.*, **36**, 535 (1922).

of surface tension and antibody formation. Indeed, it is clear that the experiments made with non-antigenic substances did not reveal any systematic disturbance (Text-Fig. 5).

This molecular phenomenon presents, between the 12th and 16th days, a maximum amplitude in all the cases reported above. After this lapse of time, a progressive decrease is observed and around the 25th day, the serum
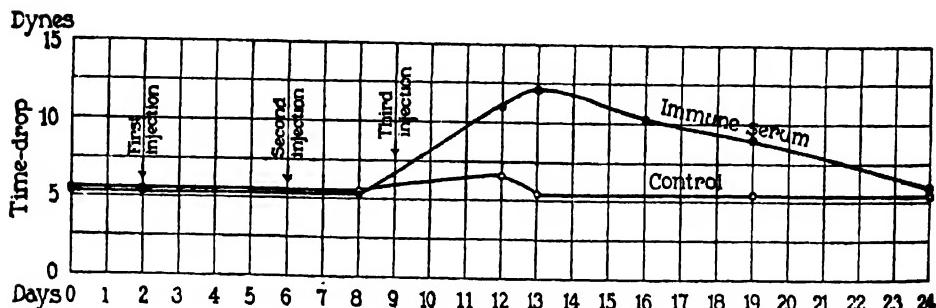
Fig. 4

seems to have returned to normal. We are too ignorant of the structure of the protein molecules even to attempt to explain these facts. All that can be done at present is to study them in their relations with the so-called antibodies so as to ascertain whether a physico-chemical manifestation due to these hypothetical substances is dealt with, or whether they are two distinct phenomena following the antigen injection. First of all, it was necessary to
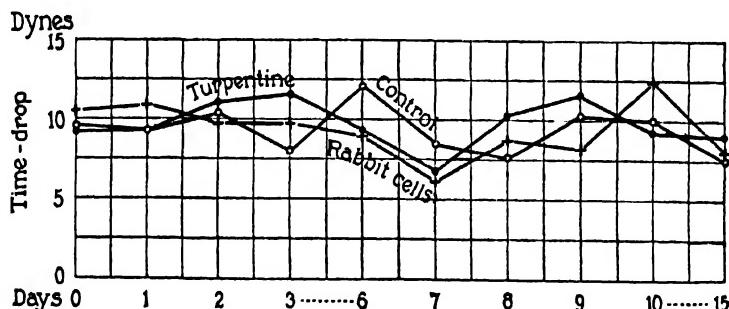
Fig. 5

determine whether the same results could be expected from bacterial immunization and from vaccine. This was proved to be the case for *Bacillus coli* and vaccine, which yielded results identical with the above-mentioned experiments.[o] The results of a series of experiments made with Dr. Noguchi's vaccine are given in Tables II and III. The immunization of the rabbits was checked by a later revaccination. An increase in the value of the time-drop, varying between 18 and 130 per cent, can be observed on the 13th day. It will be

o Lecompte du Nouy, P., *J. Exp. Med*. **40**, 129 (1924).

TABLE II. *Time-Drop of 1/10,000 Solutions of Rabbit Sera Before and After Vaccination.*

		Normal	13 Days After Vaccination	Increase in Time-Drop
		Dynes	Dynes	Per Cent
No. 1	Initial value	64.0	75.0	
	After 2 hrs...............	55.0	57.5	
	Time-drop	9.0	17.5	94
" 2	Initial value	63.0	76.0	
	After 2 hrs...............	54.5	56.5	
	Time-drop	8.5	19.5	130
" 7	Initial value	66.0	73.5	
	After 2 hrs...............	56.0	57.0	
	Time-drop	10.0	16.5	65
" 8	Initial value	69.5	76.0	
	After 2 hrs...............	58.0	58.5	
	Time-drop	11.5	17.5	52

Immune

TABLE III. *Time-Drop of 1/10,000 Solutions of Rabbit Sera Before and After Vaccination.*

		Normal	13 Days After Vaccination	Reaction	Increase in Time-Drop
		Dynes	Dynes		Per Cent
No. 9	Initial value	70.0	76.0	Good up to a dilution of 1/10	
	After 2 hours.........	58.0	61.0		
	Time-drop	12.0	15.0		25
" 10	Initial value	68.0	75.5		
	After 2 hours.........	57.0	62.5		
	Time-drop	11.0	13.0	1/10	18
" 3	Initial value	76.0	77.0		
	After 2 hours	67.2	65.5		
	Time-drop	8.8	11.5	1/10	31
" 4	Initial value	73.5	76.5		
	After 2 hours.........	65.0	59.8		
	Time-drop	8.5	16.7	1/10	97
" 5	Initial value	75.5	77.0		
	After 2 hours.........	65.5	64.0		
	Time-drop	10.0	13.0	1/100	30
" 6	Initial value	75.5	77.0		
	After 2 hours.........	66.5	66.0		
	Time-drop	9.0	11.0	1/10	22

Immune

noticed that this phenomenon is essentially non-specific, that is, the presence of totally different antibodies affects the drop in surface tension in the same way.

During the course of the preceding experiments which lasted about sixteen months, great difficulty was encountered in securing absolutely normal animals. The values of the time-drop of fresh animals supposedly normal varied between 6 and 18 dynes without any apparent reason. An animal with a time-drop of 18 dynes will show no, or very little, increase in this value if it receives an antigenic injection. The conclusion was, in the case of rabbits for instance, that an animal, even though of healthy appearance and considered to be normal but which shows a time-drop of more than 10 dynes, has antibodies in its circulation and is unfit for experimental purposes. In a lot of 25 new rabbits, frequently more than 15 had to be discarded. The eliminated ones were kept under observation and most of them showed positive symptoms of snuffles within a few days. Some of them died and yet at the time of their delivery, there was nothing to indicate the possibility of such an epidemic. Those which, although showing a time-drop of over 10 dynes, did not subsequently develop snuffles, were probably recovering from the same disease and, therefore, were also unfit for experimental purposes. From that time on, no animals showing a time-drop higher than 10 dynes were used, and the phenomenon consecutive to immunization was observed regularly.

This observation, which makes it possible to effect a rigorous selection among animals and to obtain constant results for all experiments on immunity, indicates that as far as man is concerned, the study of the time-drop will probably be unable to yield any useful information, unless very young children are dealt with.

Experiments showed, indeed, that the normal time-drop for the adult varied between 15 and 22 dynes. The serum of 67 children, from 6 months to 8 years old, treated at the Babies' Hospital in New York City,[10] gave results which may be summarized in the following way:

Time-drop in dynes	16 to 20	14 to 16	12 to 14	10 to 12	7 to 10
Number of children	33	16	11	4	2

All these children, with the exception of three, had received an injection of diphtheria antitoxin. One of those who showed a drop comprised between 7 and 10 dynes (8.7) had not yet received this injection, and the other (6 months old) had received it only three days before the bleeding. Neither one had an infectious disease. It is difficult to draw any conclusion except that it is probably impossible to find in a human being a serum entirely devoid of some kind of immunity, either natural or acquired.

Many problems arose as a consequence of the preceding experiments. Our knowledge of the phenomenon depended on their solution.

1. Did the increase in the time-drop of surface tension consecutive to the antigen injection really correspond to a decrease in the absolute value of the surface tension?

2. Except in the case where vaccine was used and simply smeared on the skin, the animals had all received from three to four injections of antigen. What would happen if only one injection were made? Would the maximum

drop occur after the same number of days or after a shorter period of time, and would the drop be of the same magnitude?

3. Is the amplitude of the drop function within certain limits of the amount of antigen injected?

4. Is the thickness of the adsorbed monolayer affected in a measurable way by immunization? In other words, is the change in the individual field of forces of every molecule accompanied by a change in the length of the molecule?

5. Would a new antigen injection given immediately after the maximum (around the 13th day) result in an increase of the phenomenon, or would it delay its decrease?

6. Could the phenomenon be repeated by injecting a new amount of antigen on the 30th day when the serum seems to have returned to normal? In other words, has the serum undergone a more or less permanent modification or a temporary one?

TABLE IV.

Day After Antigen Injection		5	6	7	8	9	12	13	15	16	19	20	21	23	26	27	28
Controls	No. 1	X		X		X	X	X		X		X		X		X	
	" 2	X		X		X	X	X		X		X		X		X	
	" 3	X		X		X	X	X		X		X		X		X	
	" 4		X		X		X	X	X		X		X		X		X
	" 5		X		X		X	X	X		X		X		X		X
	" 6		X		X		X	X	X		X		X		X		X
Experimental	No. 7	X		X		X	X	X		X		X		X		X	
	" 8	X		X		X	X	X		X		X		X		X	
	" 9		X		X		X	X	X		X		X		X		X
	" 10		X		X		X	X	X		X		X		X		X
	" 11		X		X		X	X	X		X		X		X		X

7. Could the phenomenon be due merely to the presence of antigen in the circulation?

8. Is there a change in the relative proportion of globulins and albumin on the 13th day?

9. In what way is this phenomenon related to the so-called antibodies?

These problems will be taken up successively. Several of them could be elucidated by one type of experiment. Therefore, one of these experiments will be described in detail.[11]

Twelve rabbits were chosen, six of which were used as controls. All of these animals were isolated and studied for a period of 2 months before the injection, in order to make certain that they had normal time-drops of less than 10 dynes. They were bled three times during this period and were found to be normal. The experimental animals were then injected intravenously with the antigen made from crushed horse kidney,[12] provided by Dr. Landsteiner. This antigen was chosen because of its harmlessness and its constant results. In previous work, some trouble had been experienced with other antigens. One of the animals died just before the experiment was started,

[11] Lecompte du Nouy, P., *J. Exp. Med.*, **41**, 779 (1925).
[12] The horse kidney is crushed and diluted to 1/10 in physiological solution (NaCl 0.9 per cent). 0.5 per cent of phenol is added as a preservative. The mixture is allowed to settle and the supernatant fluid is used.

TABLE V. *Dilution at Which the Minimum Value of Surface Tension Occurred in Five Experimental Animals and Three Controls.**

| | Rabbit No. | | | | | | | | Mean Value of Experimentals | Mean Value of Controls |
| | Experimentals | | | | | Controls | | | | |
Day after antigen injection:	1	2	3	4	5	6	7	8		
Mean value of measurements made before injection of antigen	10,500	10,000	10,500	10,500	10,500	10,500	10,500	10,500	.....	.....
5	10,500	.....	.....	10,500	11,000	.....	10,000	.....	10,666	10,166
6	.....	10,000	11,000	11,000	11,000	10,000	10,000	10,500	10,500	10,500
7	10,000	10,000	11,000	11,000	11,000	10,500	10,000	11,000	10,666	10,333
8	10,000	.....	.....	11,000	.....	.....	.....	.....	10,500	.....
9	10,000	10,000	11,000	11,000	10,500	10,500	10,500	11,000	10,600	10,333
12	10,000	10,000	10,500	10,500	11,500	10,000	10,500	10,500	10,500	10,666
13	10,500	10,000	11,000	10,500	11,000	10,500	10,500	11,000	10,750	.....
15	.....	10,500	.....	10,500	10,500	.....	.....	.....	10,333	10,500
16	10,000	.....	10,500	10,500	10,500	10,500	10,500	10,500	10,500	10,500
19	10,500	10,500	10,500	10,500	10,500	10,500	10,500	10,500	10,500	10,500
20	10,500	10,000	11,000	10,500	10,500	10,000	10,500	10,000	10,500	10,166
21	10,500	.....	.....	10,500	10,500	10,500	10,000	10,000	10,500	.....
23	10,500	10,500	10,500	10,500	10,500	10,500	10,000	10,000	10,500	10,166
26	.....	.....	10,500	10,500	10,000	.....	10,000	10,000	10,500	.....
27	10,500	10,500	.....	10,500	10,500	10,500	10,500	10,500	10,500	10,500
28	.....	.....	11,000	10,500	.....	.....	.....	.....	10,750	10,500
29	10,500	11,000	10,500	.....	.....	10,000	10,500	10,500	10,333	10,333
30	.....	.....	.....	.....	.....	.....	.....	.....	10,750	.....
Mean value of all experimentals									10,517	
Mean value of all controls										10,400

* Figures shown represent one part in the number stated, i.e., one part in 10,500, etc.

so only five were left in addition to the controls. Rabbit 1 received 4 cc. of the antigen; Rabbits 2 and 3, 5 cc.; Rabbits 5 and 6, 10 cc. All were kept and fed in the same way as the controls. Measurements were made every other day. Crosses indicate the time of the bleedings in Table IV.
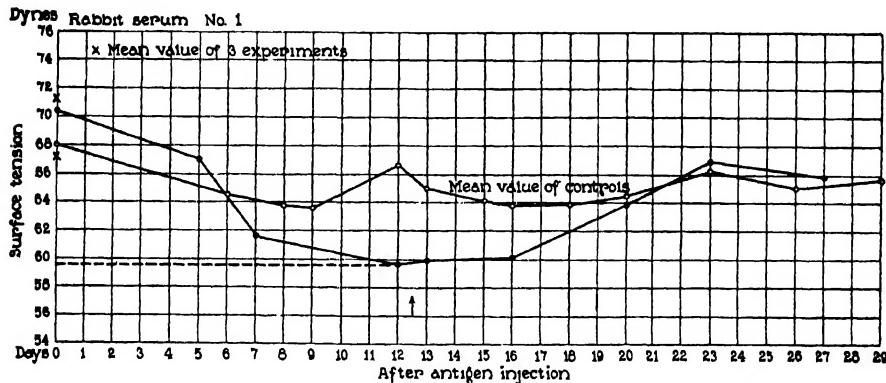
FIG. 6

It will be seen that the controls and the experimental animals were bled alternately, except on the 12th and 13th days when the time-drop phenomenon was expected to reach its maximum. The reasons for not making the measurements every day were, first, that the animals might be disturbed by too frequent bleedings, and second, because of technical difficulties. It must be
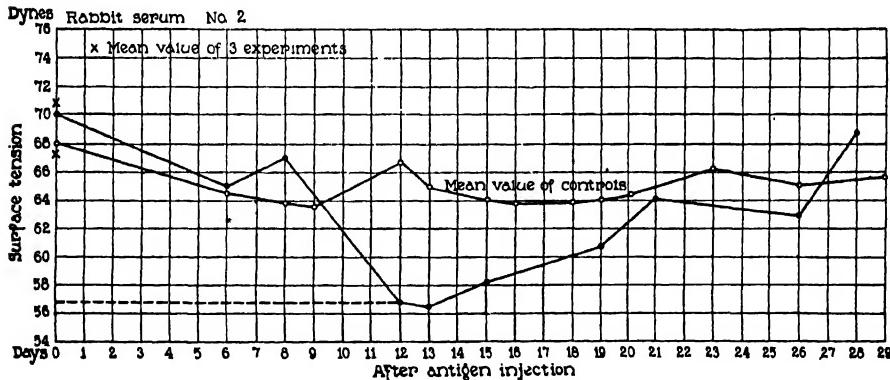
FIG. 7

borne in mind that seventeen different dilutions of the serum were made in every case. This in itself required considerable glassware which had to be cleaned with the utmost care. As two measurements of the surface tension were required at 2 hours' interval, this meant 34 measurements for each serum. When all sera were dealt with, as on the 12th and 13th days, 374 measurements

of surface tension had to be made in one day. This amount of labor can be carried out during two days, but it becomes extremely taxing on all concerned when done oftener. Fortunately, this appeared unnecessary. The
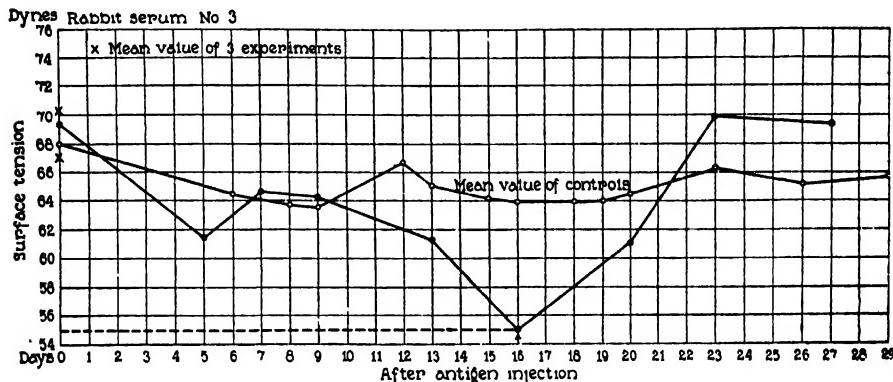
FIG. 8

first set of experiments reported here required a total sum of 4,420 measurements of surface tension. This was rendered possible through the use of three tensiometers on the tables described in previous papers.
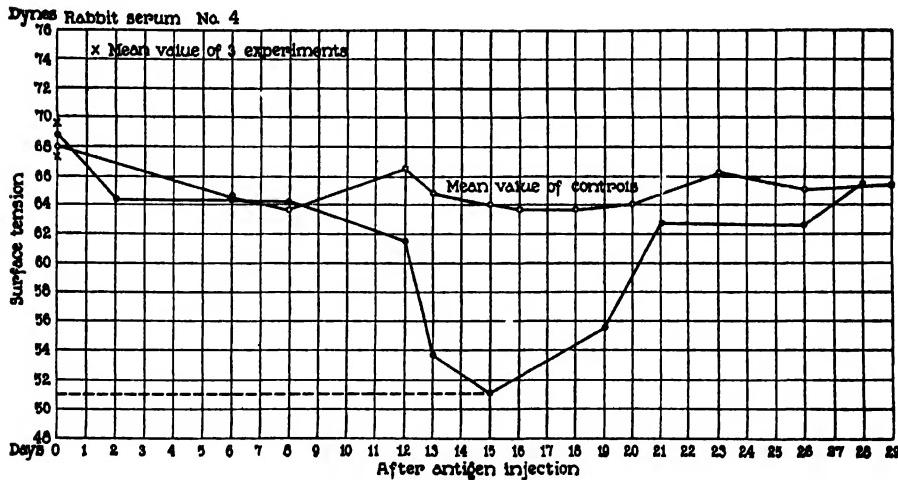
FIG. 9

The seventeen different dilutions of every serum were made because it was important to know whether the maximum drop, or absolute minimum of static surface tension, always occurred at the same dilution, or whether it was slightly shifted, perhaps as a consequence of the injection of antigen. The following dilutions were studied: 1/10, 1/100, 1/1,000, 1/7,500, 1/8,000,

1/8,500, 1/9,000, 1/9,500, 1/10,000, 1/10,500, 1/11,000, 1/11,500, 1/12,000, 1/12,500, 1/13,000, 1/100,000, 1/1,000,000. The dilutions 1/10,500 and
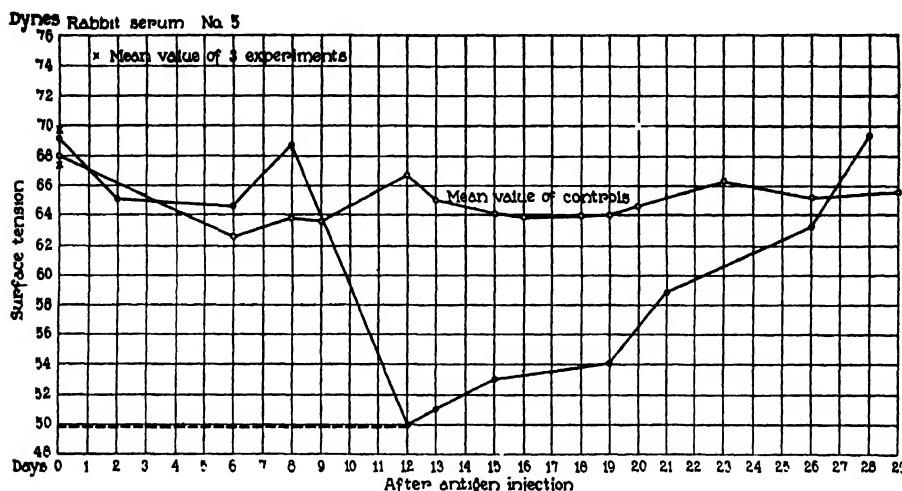
FIG. 10

1/11,000 correspond to a difference in thickness of the adsorbed layer of roughly, 2×10^{-8} cm. As the thickness of the monolayer of total rabbit serum
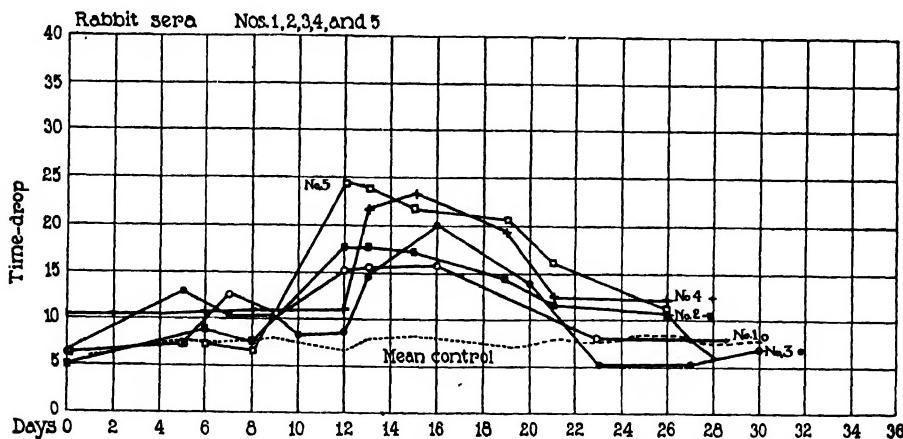
FIG. 11.—Time-drop, after 2 hours, of sera Nos. 1, 2, 3, 4, and 5, and control curve (6 controls)

is about $41 \times {}^{-8}$ cm., this means that a change in the thickness of about 5 per cent could be detected in this vicinity. On taking into account the working conditions and the variations likely to arise from changes in the concen-

tration of the proteins over a period of 3 months in the animal's life, this was considered as the maximum of accuracy that could be reasonably expected. •

Table V gives the concentrations at which the minimum surface tension was observed for the five experimental and two control animals. It is obvious that no constant shift occurs after immunization. Such differences as were noted may be laid to experimental errors. It follows that no variation greater than 5 per cent occurs in the mean length of the protein molecules after immunization. This answers question No. 4.
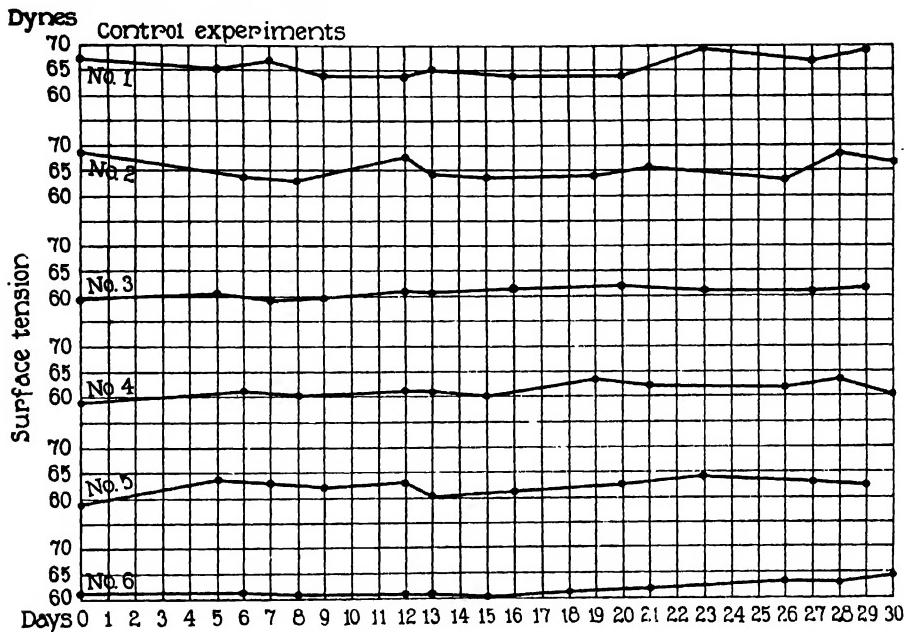
Fig. 12.—Static values, after 2 hours, of normal rabbit sera (controls), in the same conditions as the experiments reported on Figs. 1-5.

The answers to questions Nos. 1, 2, 3, 5, and 6 are found in Figures 6 to 10. Figure 11, in which the time-drop is plotted, summarizes the results.

It is clear that in all cases an absolute minimum of the static value of surface tension is observed (question 1), and that the minimum occurs practically after the same number of days (between the 12th and 16th) whether the animals receive one or four injections of antigen (question 2). It is also evident that, to a certain extent, the absolute value of the minimum seems to be a function of the quantity of antigen injected. Rabbit No. 5 reached on the 12th day the extremely low value of 50 dynes, which was less by 18 dynes than its normal static value. Rabbit No. 4 reached 51 dynes. Both had received 10 cc. of antigen (question 3). On the 15th day, they received another injection of antigen equal to the first, namely, 10 cc. No change in the curve resulted. It appears from these findings that an injection of antigen immediately after the maximum does not result in an increased phenomenon

(question 5), and that the increased time-drop is not due to the presence of antigen in the circulation (question 7).
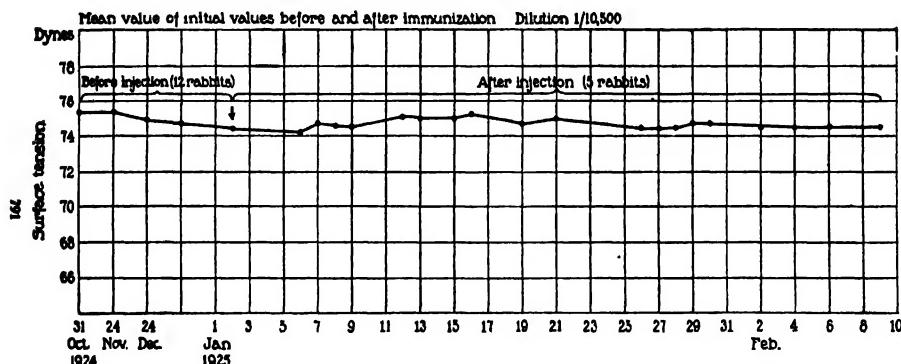
FIG. 13

Rabbits Nos. 1, 2, and 3 received a second injection of the same amount of antigen on the 30th day. They were bled 13, 14, and 15 days later, and

TABLE VI. *Proportion of Albumin and Total Globulins in Rabbit Serum.*
(In Percentage.)

Nos.	Before Immunization					After Immunization (13th day)			
	1	2	3	4	5	1	2	3	5
Albumin	69	71	78	76	71	71	69	74	73
Globulin	31	29	22	24	29	29	31	26	27
Ratio: $\frac{A}{G}$	2.22	2.45	3 50	3.16	2.45	2.45	2.22	2.85	2.70
Mean value of the ratios ..	2 76	....	....	....	....	2.56	....	....	....

Control Animals.

Nos.	Before Immunization		After 13 Days	
	1	2	1	2
Albumin	75	78	70	77
Globulin	25	22	30	23
Ratio: $\frac{A}{G}$..............	3.0	3.50	2.35	3.35
Mean value of the ratios	3.25	....	2.85	....

their sera did not show any minimum. This experiment was repeated on other animals with the same negative result.[13] The serum had consequently undergone some kind of a change of a more permanent nature (question 6).

[13] It sometimes happens that the static value at the end of the first period of 30 days is higher than at the beginning. Usually the serum retains this value for some months.

On the other hand, the amount of antibodies was increased, as indicated by the hemolytic test *in vitro*. Therefore, it seems as if the phenomenon revealed by the existence of the minimum value of the static tension is not directly correlated with the presence of antibodies, but that the two phenomena are simply coexistent at the beginning of immunization. These two manifestations of the state of immunity differ not only in their duration, but also in the fact that a subsequent injection of antigen, while it determines a recrudescence in the antibody content, does not cause a reappearance of the

PLATE 1.—A photomicrograph of the sodium chloride crystals, after evaporation of a solution at 1:10,500 of *normal* serum (× 10, in a watch glass, polarized light, blue filter).

minimum. It looks as if the serum proteins had acquired a character as permanent as immunity itself. At the present time, it is impossible to answer the ninth question more definitely.

Text-Figures 12 and 13 express the result of measurements made with the serum of the controls and the values of the initial surface tension of solutions during a series of experiments. It will be seen that this value is practically constant throughout the series. The phenomenon manifests itself only by its action on the static value of the solutions after standing 2 hours.

As far as the change in the percentage in globulins and albumin is concerned, it can be stated that the phenomenon in question is entirely independent

of it. A series of analyses were made by Dr. Baker [3] on the serum of animals immunized with *Bacillus coli*, sheep cells, egg albumin, and organ extracts. The results of these analyses showed that the ratio $\frac{albumin}{globulin}$ varies little and certainly not more in the immunized animals than in the controls. It will be seen in Table VI that, in one series of experiments, the controls showed a greater decrease of the ratio $\frac{G}{A}$ than the experimental animals.

PLATE 2.—A photomicrograph of the sodium chloride crystals, after evaporation of a solution at 1 : 10,500 of *immune* serum ($\times$ 10, in a watch glass, polarized light, blue filter).

An attempt was made to answer question 7 (action of the antigen on the serum) still more definitely. The normal serum of seven rabbits was mixed with an amount of different antigens corresponding to the quantity used for immunization.[3] The mixture was then kept in the incubator at 37° C. for 13 days. The time-drop was measured before the addition of antigen and at the end of the 13th day. No increase was observed in the value of the time-drop which remained around 10 dynes. It may, therefore, be concluded that, as is the case with immunization, the presence of living cells is required to produce the phenomenon of the decrease of surface tension.

In certain cases, the deep perturbation undergone by the molecules around the 13th day, when the phenomenon reaches its maximum, manifests itself in strongly immunized animals in a very striking way by its action on the crystallization of the NaCl in the solution. In Plates I and II, the differences observed in the structure of NaCl crystals before and after immunization are

TABLE VII. *Refractive Indices of Immune and Normal Sera the Time-Drop of Which Is Reported in Table I.*

Date	Controls		Rabbits Injected with Sheep Cells			Rabbits Injected with Egg White		
	No. 9	No. 10	No. 11	No. 12	No. 13	No. 14	No. 15	No. 16
1922								
Sept. 28	1.3438	1.3429	1.3442	1.3422	1.3440	1.3433	1.3438	1.3429
" 29	1.3398	1.3392	1.3399	1.3385	1.3402	1.3390	1.3407	1.3325
Oct. 2	1.3398	1.3386	1.3409	1.3390	1.3408	1.3397	1.3410	1.3381
" 3	1.3440	1.3387	1.3404	1.3398	1.3405	1.3405	1.3420	1.3390
" 4	1.3388	1.3384	1.3395	1.3388	1.3400	1.3398	1.3399	1.3392
" 5	1.3398	1.3393	1.3408	1.3350	.....	1.3395	1.3401	1.3385
" 6	1.3422	1.3415	1.3415	1.3410	1.3420	1.3440	1.3415	1.3420
" 9	1.3415	1.3413	1.3401	1.3420	1.3402	1.3405	1.3411	1.3405
" 10	1.3400	1.3388	1.3391	1.3402	1.3403	1.3403	1.3398	1.3391
" 11	1.3391	1.3385	1.3391	1.3383	1.3396	1.3396	1.3390	1.3386
" 13	1.3399	1.3385	Died	1.3385	1.3383	1.3390	1.3391	1.3391
" 16	1.3389	1.3385	.....	1.3370	1.3390	1.3384	1.3392	1.3390
Mean average	1.3400		1.3400					

shown. The existence of such a modification in the molecules could be expected to manifest itself by other physical phenomena, for instance, such as a variation in the refractive index. Table VII shows that this is not the case.[14]

DISCUSSION

Our knowledge of the relation between the chemical structure and properties of organic compounds, and the forces existing between their molecules or group molecules, when in solution, is too scant to permit an interpretation of the meaning of the value and changes of the surface tension of a liquid. Furthermore, our knowledge of the chemistry and physicochemistry of antibodies is practically nil. Therefore, the meaning of the experimental observations gathered in this paper remains to be discovered. But one thing may safely be said: in certain immunity states, a decrease in the surface energy of the substances normally adsorbed in function of time in the surface layer is observed. It is not improbable that the method will yield more accurate data than those furnished by biological tests in following, for example, the progress of the process of immunization.

CONCLUSIONS

An attempt was made to apply to the study of immune sera the fact, pointed out in our previous papers, that at a given dilution the serum shows

[14] For further details see: Lecomte du Nouy, P., "Surface Equilibria of Biological and Organic Colloids," New York, 1926.

a maximum drop of surface tension in function of time (time-drop). The results of the experiments here reported would seem to indicate that:

1. The time-drop of a scrum solution is greater after the animal has been immunized than before immunization (from 50 to 100 per cent).

2. The time-drop is maximum at a dilution of 1 : 10,000, as had been predicted from the data previously acquired.

3. The size of the molecules or micellae is probably unchanged after immunization, since the maximum drop is not shifted then.

4. Measurements made during the process of immunization showed that the time-drop increases after the 8th day and second injection, and reaches a maximum toward the 13th day, while the controls undergo no increase.

5. The injection of substances non-productive of antibodies, such as homologous cells or turpentine, does not result in the production of a permanent maximum. This shows that the maximum observed when heterologous cells are used is due to the presence of antibodies, and not to the mere injection of foreign material.

6. The measurement of the refractive index, of the sera failed to demonstrate any differences between the sera of the controls and those of the experimental animals.

Experimentally, the effects of colloids on crystallization have been much neglected, though the subject has been often referred to. See references in the index of this book to Wm. U. Ord's work; also J. Alexander, Kolloid Zeit. for 1909.   J. A.

Lobar Pneumonia and its Treatment with Refined Sera

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Service, Harlem Hospital, Lewis K. Neff, Director,
New York City

Bacteriological Diagnosis. Differentiation of Types

The *diplococcus pneumoniae* is the organism responsible for the majority
of cases of lobar pneumonia and less frequently for various other inflam-
matory processes.

Bacteriologists have differentiated several strains or types of pneumococcus
just as they have for many other pathogenic organisms. The types of pneu-
mococcus have been made the subject of many valuable studies especially in
the United States. The solubility of the streptococcus mucosus in bile,
aside from morphological, cultural and clinical reasons, induced William H.
Park (1905) to classify this organism as pneumococcus mucosus. Avery and
Dochez (Monograph No. 7, 1917) designated the pneumococcus mucosus,
"Type III". This type as well as the so-called Types I and II of the pneu-
mococcus show strongly pronounced specific immunity, i.e., when injected into
animals they cause the formation of antibodies of highly specific action for
each type but low in general specificity. These three types are responsible
for about three-quarters of all pneumonia infections in the temperate zone.
The remainder are due to a number of other types, some of which are quite
virulent. All the strains except the original three fixed types are usually classi-
fied as group IV or miscellaneous. Georgia Cooper working with William H.
Park has been able to select seven of these miscellaneous strains by cross agglu-
tination for specific serum production, which are responsible for over thirty
per cent of the miscellaneous group of pneumonias in New York City. Of
these, two are the sub-group IIA and IIB of the Rockefeller Hospital
workers.

As a result of the introduction of therapeutic type specific antibody solu-
tions the recognition of the type of invading pneumococcus has become of
prime importance.

"Typing" of the invading organisms may be carried out in the following
manner with the sputum.

(1) Krumweide-Valentine Test. The sputum is coagulated by boiling;
the coagulum is extracted with saline; qualitative precipitin tests of these
extracts with "diagnostic" monovalent antiserum allow the differentiation
of the type. This test should be used whenever sufficient (about 5 cc.) amounts
are available because it requires a relatively short time.

(2) The Avery test consist of intraperitoneal inoculations of a mouse
with sputum and a subsequent (after 8 hours) agglutination or precipitin
test with the peritoneal exudate. At times the organism may be recovered

by culture from the mouse heart's blood and typed. This should be done in all cases, as the fixed types may prove more invasive than the miscellaneous group.

(3) The Oliver test is a rapid method of diagnosis based on the bile solubility of organisms found in the sputum. Solution of the capsule by the bile salts liberates the soluble specific substance.

In a certain percentage of cases the type of pneumococcus invading the blood stream may be directly detected and studied by cultures. In many cases, the soluble type specific substance may be found in the urine, as well as in the sputum.

Characteristics of the antibodies mentioned above, are (a) bactericidal action, (b) agglutination of homologous cocci, (c) precipitation with the soluble specific carbohydrates of Avery and Heidelberger. These carbohydrate substances have been proven to be gum-like polysaccharides with carboxyl functions. From each of the three fixed types a soluble specific substance of definite chemical individuality has been isolated, (d) antihematoxic.

The presence of antibody in the blood stream as the result of either active or passive immunization may be tested by the properties of these antibodies, enumerated in the preceding paragraph. Sometimes the presence of free soluble specific substance has been observed in the blood.

PREPARATION OF ANTIBODY SOLUTIONS

The antibodies solutions have been prepared by several methods; usually killed organisms are injected. The anti-pneumococcic serum is as a rule obtained from horses though Preston Keys obtained it from fowl. The serum is called monovalent or polyvalent, depending on whether a single type or more than one type of pneumococcic culture is injected into the animal.

Any preparation of antibody solution has as its aim two equally important fundamental purposes; concentration and purification: only simultaneous achievement of both objects will mean real progress. A maximum yield must be obtained if the serum is to be generally employed. Besides these problems met with in any preparative work on physiologically active substances such as hormones, enzymes or vitamines, there is here a special problem of purification. Besides general purification, i.e., removal of inert substances, it is necessary to eliminate unknown substances which are harmful to patients. Some of these substances produce chills and at the same time depress the number and activity of leucocytes and cause vacuoles to appear in them. The following are the more important procedures tried in the case of antipneumococcic serum.

SEPARATION OF CERTAIN PROTEIN FRACTIONS

This involves the separation of certain fractions of serum proteins with an increased antibody titer as compared both with the titer of the original serum per cc. (concentration) and per unit of total solids sometimes expressed in milligrams of nitrogen (purification). The standard reagents for the separation are $NaCl$, $(NH_4)_2SO_4$, $MgSO_4$, Na_2SO_4, and related salts. Although the terminology of the serum proteins varies among different schools, the protein fraction soluble after 50 per cent saturation with ammonium sulfate is generally known as the serum albumin. It constitutes about 2 per

cent of the serum and has often been demonstrated as free from antitoxins and antibodies. The same may be said concerning the fibrinogen of the plasma.

The globulins can be separated in various ways and apparently these methods draw various cross sections through the groups involved though they overlap to a certain degree. The less soluble half of the entire globulin fraction is usually designated as euglobulin, the other one as pseudoglobulin. Saturation with NaCl will carry down the euglobulins; 25 to 30 per cent saturation with ammonium sulfate is supposed to precipitate the euglobulin also, but there is little evidence for the identity of this fraction with the euglobulin insoluble in NaCl. A fraction is separated by definite concentrations of the other sulfates mentioned above which is closely related to that separated by ammonium sulfate.

By diluting with distilled water, the electrolyte content of the serum can be reduced to a fraction of the physiological titer (0.9% as NaCl). The fraction rendered insoluble by this treatment has also been called euglobulin.

One innate and two external factors still complicate matters at this point. Immune sera have apparently not the same proportion of the protein fraction as have normal sera. In the case of antitoxic sera (foremost Diphtheria) but also to a lesser degree in that of anticoccic sera there is an absolute increase of total proteins: normal sera contain 6 to 7 per cent; immune sera markedly more, sometimes as much as 12 per cent total solids and 11 per cent proteins. This increase is largely due to an increase in pseudoglobulins. The percentage of albumin decreases relatively and sometimes even absolutely. The euglobulin according to its first definition (insolubility in salt solutions) is actually decreased. But if defined by its solubility in very dilute electrolytes, then the euglobulin fraction is found to be increased, as the result of immunization.

The two external factors which can be arbitrarily adjusted are (1), the pH; (2), the presence of certain antiseptics or preservatives.

(1) Since the normal pH of serum is very nearly that of the blood the proteins are decidedly on the alkaline side of their isoelectric points (4.5-6.0). When the isoelectric zone is approached by lowering the pH, they generally become less soluble and smaller amounts of salts will suffice for the precipitation of a given fraction.

(2) The presence of preservatives such as phenol or tri-kresol in concentrations of a few tenths of a per cent will increase the "salting out" power of certain salts to a surprising degree.

Banzhaf, in accordance with Avery attained considerable concentration and satisfactory yields by selecting a certain pseudoglobulin fraction, roughly speaking, that obtained by saturation with between 30 and 40 per cent ammonium sulfate. This procedure could be further improved by precipitating these pseudoglobulins a second time by addition of distilled water at lowered pH and redissolving this precipitate in physiological salt solution.

The treatment of the original serum with distilled water at lower pH, sometimes referred to as "Felton's Method", has been practiced by the Boston scientist and the results are as satisfactory as by the other method.

Since these methods lead to similar results in titer (2000 protective units for Type I) and purity, but without wholly eliminating the unwelcome production of chills after intravenous injections, it was obvious that each of the

two preparations still contains inert and even undesirable substances, although not necessarily of identical nature.

Felton by fractionate precipitation with salts, as, *e.g.,* nitrate and varying amounts of acid, eliminated a "first euglobulin" fraction containing 20 per cent of the antibody. An appreciable amount of antibody was found in the "second euglobulin" fraction. The first fraction contains a much higher proportion of organic phosphorus as compared with the nitrogen than the second. The elimination of some phosphatide contained in the first fraction may account for the infrequency of chills in the therapeutic application of the second fraction. Felton processes the serum at ice-box temperature, in order to remove certain lipoids.

Banzhaf and Sobotka, by trying numerous combinations of salts, developed a short-cut method which can be carried out in slightly diluted sera with a few operations. Starting from a serum with 400 protective units they obtained a preparation of 6000 units, *i.e.,* a 15 times concentration both per volume and per total solids. The clinical reports on these products are favorable.

Some work is in progress on the adsorption of anti-body on aluminium gels, and subsequent elution.*

IMMUNOLOGICAL METHODS OF PURIFICATION

Immunological methods for the purification of pneumococcus antibodies have been tried with little success up to the present time. Their principle is fundamentally sound and should be discussed, since they comply with the most modern trend of preparative chemistry, namely to use gentle means in the isolation of natural products.

The antibody can be precipitated by the precipitinogen (soluble specific carbohydrate). The precipitate from the serum consists of the antibody, both the precipitin antibody and the protective antibody. (The question whether there are various antibodies with single functions or one single antibody exerting various functions, will not be discussed). These precipitates and their dissociation products have been tested in protection experiments by Heidelberger, Felton and others with inconsistent results.

A similar procedure is used by F. M. Huntoon. He uses the entire antigen. *i.e.,* the pneumococci themselves, for adsorption of the antibodies from serum presumably by their agglutinative function. The agglutinated bacteria are then washed free from serum and resuspended in certain salt solutions, *e.g.,* carbonate, and dissociated at higher temperatures. After removing the bacteria by centrifugalization an antibody solution is obtained of very low total solids and nitrogen. The high protective power claimed could not be verified by some other workers. Besides untoward reactions seem to be more frequent with this than with any other kind of antibody preparations. This may be due to the presence of small amounts of bacterial proteins. It seems that this highly purified solution of antibody is very unstable particularly when diluted. It can be concluded from the mere possibility of obtaining this kind of an antibody solution and also from some facts mentioned below that the antibody proper is not a protein itself but usually associated with certain globulins acting as protective colloids and separation from the latter may lead to gradual inactivation. (Similar conditions are known to prevail in enzymes).

* See paper by R. Willstätter, in this volume. *J. A.*

STANDARDIZATION OF ANTIBODY SOLUTION

The standardization of antipneumococccus serum antibody preparations and generally the quantitative determination of antibody in all kinds of solutions during their preparation and during their therapeutic use can be carried out in different ways:

Protective tests are carried out on mice. Either the amount of antibody or the amount of bacterial culture (Rockefeller Institute method) may be varied, the other factor being kept constant. The protective units are finally expressed in the relation of cc. antibody solution to number of minimal lethal doses in the combination just sufficient to delay the death of a mouse over 96 hours after both factors have been simultaneously injected into the animal.

The amount of antibody protecting against 1 million minimum lethal doses is one protective unit. If one two-hundredth (0.005) cc. of a given preparation contains one such unit the serum will contain 200 units per 1 cc.

Although these tests furnish direct evidence concerning the protective potency of a serum they have numerous disadvantages such as the long time required, need of uniform animals and cultures, lack of accuracy dependent on the number of dilutions and mice used, etc.

A quantitative precipitin test actually offers many advantages, but does not supplant the use of mice. The precipitin reaction lends itself more readily to quantitative investigations than the agglutinin reaction because it eliminates the use of unstandardized cultures. The precipitin test as carried out by Sobotka and Friedlander was primarily developed for the standardization of horse serum, but it may be modified and applied for diagnostic and clinical purposes. It can be carried out by combining various dilutions of antibody (precipitin) with varying dilutions of soluble specific carbohydrate (precipitinogen) and determining those dilutions limiting the positive range of the reaction. These limiting dilutions follow approximately a simple hyperbolic law: dilution of antiserum $\times$ dilution of precipitinogen $=$ C (constant) for a wide range of serum dilutions. One millionth of K is used as precipitin index for practical purposes. Zonal phenomena interfere with this regular behavior and have to be eliminated by taking an average over a wide range of concentrations. A moderate excess of serum (antibody) will cause fluffy, finely divided precipitate. A larger excess will make the precipitate disappear: Prozone. Excess of the soluble specific carbohydrate will yield a heavy precipitate consisting of individual floccules (*cf.* Morgan, *J. Immunol.,* 1923) a great excess will prevent the formation of any precipitate: postzone. These zones are accentuated by changes in pH, the prozone by increase, the postzone by decrease of the pH: a very tempting problem for colloidal chemists. The prozone, *i.e.,* the failure of a precipitate to appear in a mixture of antigen with excess of antibody is also enhanced by the addition of non-specific serum-constituents such as inactive globuline fractions or normal serum. The same factors have been observed to render the precipitation more sensitive in its normal range. Some refined antibody solutions of good protective power did not show any precipitin power at all unless a certain amount of these "third factors" was added. This seems to indicate the presence of some specific "co-precipitin" and at the same time points to the possible non-protein nature of the antibody. Similar conditions are found in agglutinin tests on pneumococci.

The quantitative usefulness of the precipitin test is based on the paral-

lelism between Precipitin Index and Protective Units. The quotient $\cdot\frac{P.I.}{P.U.}$ varies from 2 to 5 for Type I, is slightly lower for Type II and ten times higher, *i.e.,* ten times more relative precipitin power with Type III. These quotients hold for original sera and some stages in the preparation of refined solutions. They become unreliable beyond a certain step in the purification and they show deviations in the first periods of immunization of serum horses as the various antibody functions are not developed at parallel speed.

The electric potential of various types of pneumococci and its relation to their virulence has been discussed by Falk.

The therapeutic evaluation of anti-pneumococcic serum has been studied by the author in association with Dr. Milton B. Rosenbluth at Harlem Hospital. In a presentation recently made at the N. Y. Academy of Medicine we stressed the importance of a controlled series of cases, the data necessary about the cases before judging the effect of serum and the size of the series requisite.

The Importance of a Control Series of Cases

The evaluation of the effect of any therapeutic procedure in pneumonia is attended by certain inherent difficulties. Probably seven of every ten patients recover regardless of treatment, and therefore if one chances upon a succession of favorable cases, he is apt to attribute the benefit to the special treatment then in use. A short series of fatalities, unless carefully controlled and analyzed may lead to a condemnation of what is really a very useful procedure.

Refined concentrated serum is desirable in order that the dose may be small and readily administered without severe reactions. It is desirable that it be polyvalent (several sera may be mixed) because the pneumonias resulting from pneumococci of different serological types may be indistinguishable at the onset. Because no one can foretell with certainty the cases which may develop adequate protection in response to the pneumonic invasion, the benefit from treating patients is best discovered by comparing the results in a series of serum treated cases with a similar series treated without serum.

At Harlem Hospital each alternate patient with pneumonia is placed in the serum series. It is not practicable to alternate the cases according to type on admission as this might occasion a delay of many hours or days. The injection of a powerful polyvalent serum of Types I, II and of a less potent Type III assures prompt treatment of the cases selected for serum.

We accept and retain as pneumonia patients those having pneumococcus infections of the lung with definite lobar involvement, as evidenced by unmistakable physical signs, fluoroscopy, or radiography.

Except for the serum, all ·patients are given the same medical care in respect to drugs and nursing, in accordance with a definite plan.

Data Necessary Before Judging the Effect of Serum

On admission cases are rated in accordance with a definite plan. The rating assumes 100 to be health and for each of five categories a maximum of 20 may be subtracted.

Respiratory: Involvement. Rate. Pleurisy.
Nervous Condition: Headache. Irritability. Sleeplessness. Delirium.
　　Apathy. Coma.
Circulatory Efficiency: Rate. Cyanosis.
Gastrointestinal: Distension. Vomiting.
Complications. Special Factors.

In our series studied during the past year we had 401 cases of lobar pneumonia, of which 28 were rejected because it was impossible to type them, and 8 because of concurrent febrile diseases, such as scarlet fever, tuberculosis, etc.; 365 remain to be studied, 169 in the serum series and 196 in the controls; 220 were rated.

In a general way the rating on admission indicated chances for recovery. The Type I cases treated were sufficiently numerous to classify as good, fair and poor. Chart (5). Of 6 patients rated poor (below 50) we saved 2 patients with serum, while we lost all treated without serum; of those rated fair (50 to 70), we saved 4 of 6 in the serum group against 6 among 11 without serum. We saved with serum some very ill patients who might have died without it.

Many have maintained that the excellent results reported with serum treatment in hospitals have been due to the early hospitalization rather than to the serum. For both Type I and Type II serum series the mortality among both early and late cases was less than for the controls.

Not only did the serum treatment appear to reduce the number of deaths but it shortened the illness of those who recovered. Certainly this was advantageous, as it meant a saving in hospital care and of subsequent invalidism.

With serum we not only saved more patients and shortened the illness of those who recovered, but apparently the serum delayed death in those who perished, just as occurs in animal experiments.

When we combine all the cases early and late by types, and study the effect of the serum, it will be noted that Type I and Type II for which we had potent sera, showed fewer deaths per hundred among the serum treated cases. The greater benefit from early treatment has been revealed on charts already shown.

Size of Series Requisite

What test can we apply to our data to see whether proof is adequate and how can we determine the number of cases necessary for a judgment? This leads us into a brief digression into what differences in results is statistically significant, and the meaning of the standard error.

Without going into technical details, it may be explained that the relative spread, or flatness, of the curve of occurrence of a quality is measured by what is called the standard error of the measurements. In order that the difference between measurements in two separate materials, *e.g.*, case fatality of two similar hospital populations with pneumonia, one of which received serum, shall be recognized as definitely significant, the distance between the peaks of the two curves must satisfy a certain statistical test. The difference between the average measurements in the two cases must be at least equal to twice the "standard error" of the difference. Whenever this ratio falls

blow 2, we are not in a position to judge whether any significant meaning is to be attached to the difference.

Deaths of Pneumonia Patients Treated (a) with Serum and (b) without Serum.
Harlem Hospital, September, 1926, to October, 1927.

(Deaths within 24 hours of admission excluded.)

Type	(a) With Serum			(b) Without Serum			Difference in Case Fatality (a-b)	Ratio of Difference to Its Error
	Cases	Deaths	Deaths per 100 Cases	Cases	Deaths	Deaths per 100 Cases		
I	55	10	18±5.2	53	18	34±6.5	−16±8.3	1.9
II	26	6	23±8.3	38	14	37±7.8	−14±11.4	1.2
III	24	11	46±10.2	17	7	41±11.9	5±15.7	.3
IV	54	7	13±4.6	82	7	9±3.2	4±5.6	.7

In the Type I cases we have practically obtained a result which is twice the standard error, 1.9. A greater difference in the percentage recovery of treated cases than those untreated may be accomplished by future improvements in the serum, and by earlier administration. Rosenblüth studied the relation of bacteriemia to prognosis and serum therapy, and William H. Park and Georgia Cooper discussed the dosage. At present at Harlem Hospital the presence of antibody in the blood serum is determined by agglutinating the serum with antigen in various dilutions as a guide to treatment. Some work with precipitins using both autolyzed antigen, and soluble specific substances have given useful results.

Rosenblüth reported as follows. Comparing the mortality in patients with positive blood cultures and with negative blood cultures we found that in Type I, 16 per cent of the negative blood culture cases died while 81 per cent of the positive blood culture cases died. In Type II, 21 per cent of the negatives died while 75 per cent of the positives died. In Type III, 17 per cent.

Furthermore, the incidence of positive blood cultures in the fatal cases was more than twice as great as the incidence in the recovered cases.

Early in our work we were strongly impressed with the fact that our statistics taken in the usual manner, comparing mortality rates for treated and control cases, by type, did not adequately express the efficacy of the serum as it appeared to us at the bedside. We had often observed in patients who appeared very profoundly stricken, the most impressively favorable results. This observation and the common knowledge that serum will frequently render the blood stream free of bacteria led us to inquire more closely into the relative effectiveness of the serum in the cases with bacteriemia and in those without it. We hoped in this way to obtain more exact data as to the indications for treatment, and as to the better selection of those cases in which serum was apt to be most effective.

On comparing the results of our treated cases with those of our untreated, we found that in Type I the mortality of untreated cases, which was 37 per cent, was reduced in the treated cases to 21 per cent, a reduction of 43 per cent. In Type II the mortality of untreated cases, which was 40 per cent, was reduced in the treated cases to 26 per cent, a reduction of 35 per cent. While these figures are impressive, they do not appear to have been suffi-

ciently convincing even in Type I cases, to interest the medical practitioner in the use of this form of therapy.

In the cases with positive blood cultures, the effect of serum treatment appears to be much more strikingly shown. In Type I, 81 per cent of the untreated cases died, whereas 39 per cent of the treated cases died, a reduction in the mortality of 51 per cent. In Type II, 75 per cent of the untreated died, while 38 per cent of the treated died, a reduction in the mortality of 49 per cent, which is almost as great as that effected in Type I.

The Method of Administration and the Dosage

Park and Cooper urged that if a serum is available that can be safely given intravenously, then it should undoubtedly be so given and the whole amount of antibody be made immediately available instead of waiting for its gradual absorption which requires a period of one to two days for its completion. If, however, one is afraid to give the refined or unrefined serum intravenously, it is useful but less so if given intramuscularly.

The first injection should be large, but before we can speak intelligently of dosage we must agree on a unit of antibody. This we have done with other sera where it was possible to adopt standardized units, as in diphtheria and tetanus antitoxins. No intelligent person would now approve of giving the dose of diphtheria antitoxic globulin solution as so many cc. instead of so many units.

During the past year the Felton polyvalent preparations which we have used have varied as follows:

Type I antibody between 300 and 2000 units per cc.

Type II antibody between 100 and 1000 units per cc.

Type III antibody between 10 and 200 units per cc.

A unit is the amount which protects mice from an average of a million minimal fatal doses of very virulent pneumococci.

In toxic cases of pneumonia the antibody matching the type of pneumococcus causing the pneumonia is quickly neutralized, partly or wholly, by the specific soluble substance while the antibodies in polyvalent sera specific for the other two types are but slightly affected and decline slowly as in a normal person. The amount to be given a case of lobar pneumonia depends on the duration and severity of the disease, as these indicate in a general way the amount of toxic or immune substance probably present in the body.

Any case in which a pronounced bacteriemia has developed will need much larger amounts than one in which the blood is sterile. This is the reason why a late severe case needs as a rule much more than an early severe case. The amount of antibody needed in some cases is very moderate in others it is large and in a few it is beyond our possibility of giving.

In order to clarify our ideas as to the amount of antibody required to neutralize all the pneumococcus specific poisonous substance, blood was taken from eleven late toxic cases to be tested by Miss Cooper. Five of these were Type I pneumonia cases and six Type II. Of the Type I cases, in one, there were traces of antibody in the serum; in one, there were traces of specific toxic substance;* in two, the toxic substance in 1 cc. of each of their bloods neutralized 1 unit of antibody and in the fifth, 1 cc. neutralized 5 units. This

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* The toxic substance referred to is the soluble specific substance neutralized by antibody (Personal communication from Dr. Wm. H. Park).

would mean that in two of the toxic cases we would require about 10,000 units and in the most toxic cases about 50,000 units to neutralize the specific toxin in the body at the time of giving the injections.

In the six late and very sick Type II cases, the blood of two contained but a trace. In the blood of two others the toxic substance required one unit of antibody per cc. of blood and each of the remaining two required the enormous amount of 50 units of Type II antibody.

The dosage required for the two most toxic cases would be approximately 500,000 units, which would require 500 cc. of our present Type II refined serum. These cases entered the hospital late and had septicemia; one did and the other did not receive serum and both died.

Our opinion as to dosage is as follows: Every case should receive on admission as nearly as possible 10,000 units of Type I antibody and of Type II antibody.

Concretions

By Professor Dr. H. Schade,

University of Kiel *

It is difficult to give a definition of *"concretion"* because this term is used, rather loosely, to designate formations which in chemical composition, outward form, and inner structure may differ widely from one another. All concretions, however, have this in common: they are formed from solutions, and consist of masses, more or less compact; and though they are sometimes of quite definite shape, yet the shape is not definite in the sense in which we use that term when speaking of true crystalline aggregates. Indeed, a peculiarity of their structure is that it is entirely different from that of true crystals. Concrements are found very widely distributed not only in inorganic nature, but also in the juices of plants and of animals, including man.

I. Gall Stones and Urinary Concretions

It is from the medical point of view that we shall begin our explanation of the formation of concretions. The interest of physicians has long been directed principally to their genesis in the biliary and urinary tracts. Two typical examples of such concretions are here shown as they are found in the affected organs.

These stones lie in the cavity itself, and have no connection with the surrounding tissue. They are, in their characteristics, purely *avital* structures, that is to say, they are not a part of the organ except insofar as the tissue cells furnish the material for the formation of the stone, or the concretions in some way exert an influence on the constitution of the body fluid or on the length of its sojourn in the body.

The generally accepted theory of the origin of concretions is that *supersaturation* exists in the solution. The clinical conditions which determine supersaturation of the bile and the urine, will not be discussed here. We shall confine ourselves to the question, important from the medical point of view, of the

Fig. 1.—Gall-bladder opened to show gall-stone *in situ* (after H. Schade).

conditions of concentration and supersaturation, for a particular substance, at which precipitation may be expected to begin. Much painstaking, though inconclusive, work has been done on the medical side of this important question.

* Translated by John M. Connolly, M.D , Ph.D., LL.D., New York.

The determination of the limits of solubility in water for the separate substances has been made. But the figures thus obtained do not hold for the body-fluids, for there must be taken into consideration the influence of other substances present in the solution. The systematic application of the laws of solution and the conditions which, according to the classical doctrine, influence solubility in ordinary solutions, has failed to lead those most careful workers, W. His and Th. Paul,[1] Gudzent,[2] and others, to the desired goal.

Furthermore it became evident that there is but little correspondence between the conditions which obtain in supersaturated states of the body fluids, and those found in aqueous solutions. In the first place colloid chemistry had been able to make clear the actual discrepancies if not quantitatively, yet at least in kind and direction. For even in the case of simple aqueous supersaturation with crystalloid substances, colloid processes are operative in the stages preliminary to precipitation. In uric acid or urate supersaturation, the changes leading to separate precipitation of the solutes have become clearly understood. As the stage of visible precipitation approaches, an ordinary solution first proceeds gradually to change in the direction Ions $\rightarrow$ Molecular $\rightarrow$ Molecular Aggregate $\rightarrow$ Colloid $\rightarrow$ Clumps which settle out (H. Schade).[3] At the same time the importance is shown of the stabilizing power of the intermediate colloid phase in precipitation. The stability of colloidal uric acid has a sharp optimum between $[H] = 10^{-7}$ and $10^{-5\cdot5}$. If the reaction of uric acid or urate supersaturated solutions is beyond these limits, then the process of precipitation approximates closely to the laws of pure solutions. If, however, the reaction is within these limits of optimum colloid stabilization, the laws of pure solution do not apply. The uric acid now suddenly shows its "capricious disposition," as the

Fig. 2.—Dissected kidney with urinary concretions in the pelvis of the kidney (after L. Aschoff).*

earlier writers call it, stubbornly to remain in solution; that is, the above-mentioned change of the solute is, in consequence of the favorable stabilizing condition for the colloid, delayed; and complete precipitation takes place long after the last phase preliminary to precipitation is clearly visible to the eye.†

It is evident that in general the importance of such a colloid intermediate phase must be proportionate to the conditions favorable to its stabilization. When a colloid is added to a solution, it is able in the most manifold ways to alter the degree of this stabilization, usually to increase it, but occasionally even to lower it. In all body fluids colloids are present in more or less abundance, and generally in continually fluctuating quantity, so that we may easily

* From L. Aschoff, "Pathologische Anatomie," Vol. II, p. 504 (G. Fischer, Jena).
[1] *Z. physiol. Chem.*, **31**, 1 (1900).
[2] *Idem.*, **56**, 150; **60**, 25, 38, **63**, 455.
[3] *Z. Klin. Med.*, **93**, 1 (1922); see also *Z. physiol. Chem.*, **83**, 347 (1913).
† This seems to be an instance of *autoprotection* J. A.

understand how difficult it is to specify in precise figures the concentration at which a substance in the bile or the urine will precipitate because of supersaturation. The investigation of the problem of supersaturation in the urine and in the bile is one of the most important in the domain of colloid chemistry. But even in the study of supersaturation in pure aqueous solutions, colloid chemistry must be taken into consideration. The influence of the colloid preliminary phase upon precipitation, at least upon the time factor of events, must always be reckoned with.

Precipitation from supersaturated solutions is, however, in no way sufficient to produce concretions in body fluids. In the urine and in the bile, the ordinary sort of precipitation tends usually, not to concrement formation, but to the formation of "sediment," that is, to the existence of countless very small separate crystalline or amorphous particles which, interspersed and enveloped by pre-existing mucus or other colloid material,[4] either remain suspended in the fluid-containing cavity, or sink to the bottom of it as a light mobile jelly.

What, now, are the particular conditions which, in addition to precipitation due to supersaturation, break down this physiological protective action of mucus, and instead of loose sediment, cause firmly united concretions in the bile and in the urine? The answer to this question may best be given after first making a classification of concretions, which, I grant, is merely schematic but, nevertheless, shows clearly the essentials.

We shall distinguish:

I. Pure Colloid Concretions: Example, pure albumin concretions.

II. Pure Crystalloid Concretions: Example, pure cholesterin gall-stones; pure uric acid urinary calculi.

⎱ Originating without inflammation.

III. Mixed Colloido-Crystalline Concretions: Example, kidney and gall-stones of inflammatory origin.

This classification is in accordance with the results of the systematic research of H. Schade, in which he analyzed on a physico-chemical and especially a colloid chemical basis, the special phenomena and the general laws of concrement formation.[6]

The simplest relationships are those of the first group, of which *"pure albumin stones"* may be taken as an example. These concretions are formed whenever the fluid within a body cavity contains a considerable excess of irreversibly precipitable colloid (fibrin and other albuminous substance) without being at the same time supersaturated with crystalloid material, and this albuminous colloid, instead of attaching itself to a wall of diseased tissue,[*] has the opportunity of being deposited on some body which lies free in the cavity and is thus accessible as an adsorption center.

The combination of these conditions in the body fluids is not easily realized, and consequently the rarity of the occurrence of pure albumin stones is comprehensible. Despite their rarity, however, these concrements are very important here because they are typical examples showing how hydrophilic colloids, by their irreversible precipitation, are formed into a united mass.

[4] Within the crystalline portion of the sediment, colloids are also present as "organic scaffolding" (W. Ebstein, C. Posner, and others).

[6] H. Schade, *Munchener medic. Wochenschr.*, Nos. 1 and 2, (1909); *Z. exper. Path. Therap.*, **8**, 92 (1910); No. 14 (1911); also *Kolloid-Z.*, **4**, 175 and 261 (1909); *Kolloidchem. Beihefte*, **1**, 375 (1910); see also *Z. klin. Med.* (1922) and H. Schade, "Physikalische Chemie in der inneren Medicin," Th. Steinkopff, Dresden and Leipzig, III Edition, **1923**.

[*] The healthy mucous membrane remains free from adsorptive stratification by reason of its extremely low interfacial surface tension as compared with that of the fluids of the body cavities.

Figure 3 shows, in natural size, an albumin stone, which in the fluid of an inflamed ventral cavity has used as its nucleus a separated particle of tissue. In Figure 4 is shown the stratification of such a stone as it appears microscopically.

The usual type of deposition of an albuminous colloid is by concentric stratification. This is obvious both macroscopically and microscopically.

Fig. 3.—Pure albumin stone of the ventral cavity, after H. Schade. (Natural size.)

For the second group, that of the *"pure crystalloid stones,"* the conditions of their origin are quite different and in many ways exactly opposite. These formations occur when conditions make possible a *simple drop-wise separation* of the crystalloids, that is when the material is present in excess of the saturation point of the given solution and thus a marked *guttulate separation* is possible; and, secondly, the confluence of the droplets is not disturbed by too great an interference from the adsorption of foreign substance. Both in the bile and in the urine are found concrements formed in this manner, by pure guttulate separation.

In the bile occur the *"pure cholesterin stones"* which represent this type. Cholesterin, insoluble in water, is held in solution in bile up to a high concentration by the cholates present. Excess of cholesterin usually is thrown down as a precipitate from a pure cholate solution. But on slight addition of a fatty substance (fat extracted from bile, or olive oil, benzol, etc.) to the cholate solution, a decided change immediately takes place. We now have a separation of a well-marked guttulate character; at first of very small droplets, hardly visible microscopically, which by continual aggregation grow to balls of several millimeters in diameter. For an easy demonstration of this change in character of the precipitate resulting from the admixture of of a small quantity of clear, soluble fat, we may use, instead of aqueous cholate solution, an alcoholic solution of cholate or even an alcoholic soap solution, in which, with the aid of heat, the cholesterin can easily be dissolved, so that we have supersaturation at room temperature. As an example of a solution suitable for this purpose, take:

Fig. 4.—Pure albumin stone of the urinary bladder, after L. Lichtwitz. (Photomicrograph of stratification at circumference.)

3.5 cc. 96 per cent alcohol
1.5 cc. officinal tincture of soap
2.3 g. cholesterin

with and without 1 drop of bile, fat or olive oil.
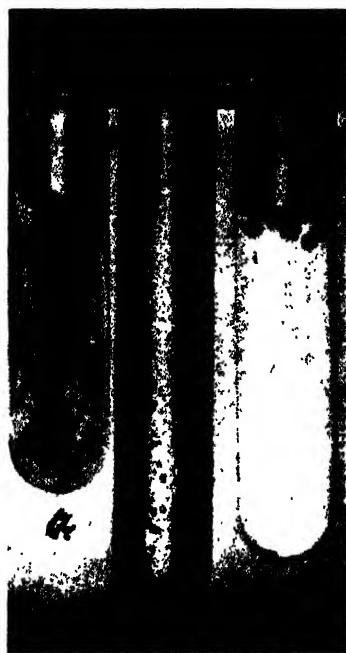
FIG. 5.—Difference between crystalline precipitation (*a*) and guttulate separation (*b* and *c*).
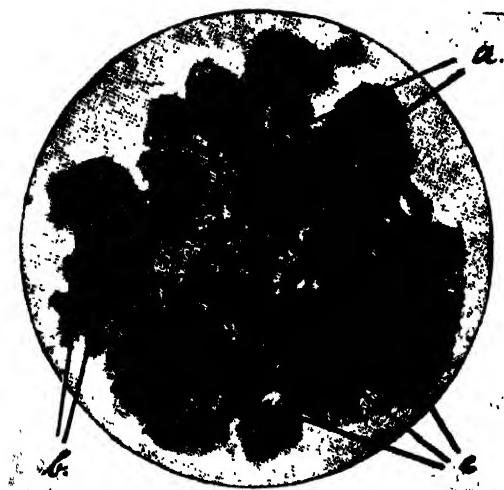
FIG. 6.—Artificially produced guttulate separation of cholesterin, after H. Schade. Microphotograph. (Enlarged 25x.)

FIG. 7.—Natural cholesterin concretion, 7 mm. in diameter. From the bile, after B. Naunyn. (Enlarged 7x.)

Figure 5 clearly shows in tubes a and b the difference between these two methods of precipitation.   In tube c are to be seen floating on the surface of
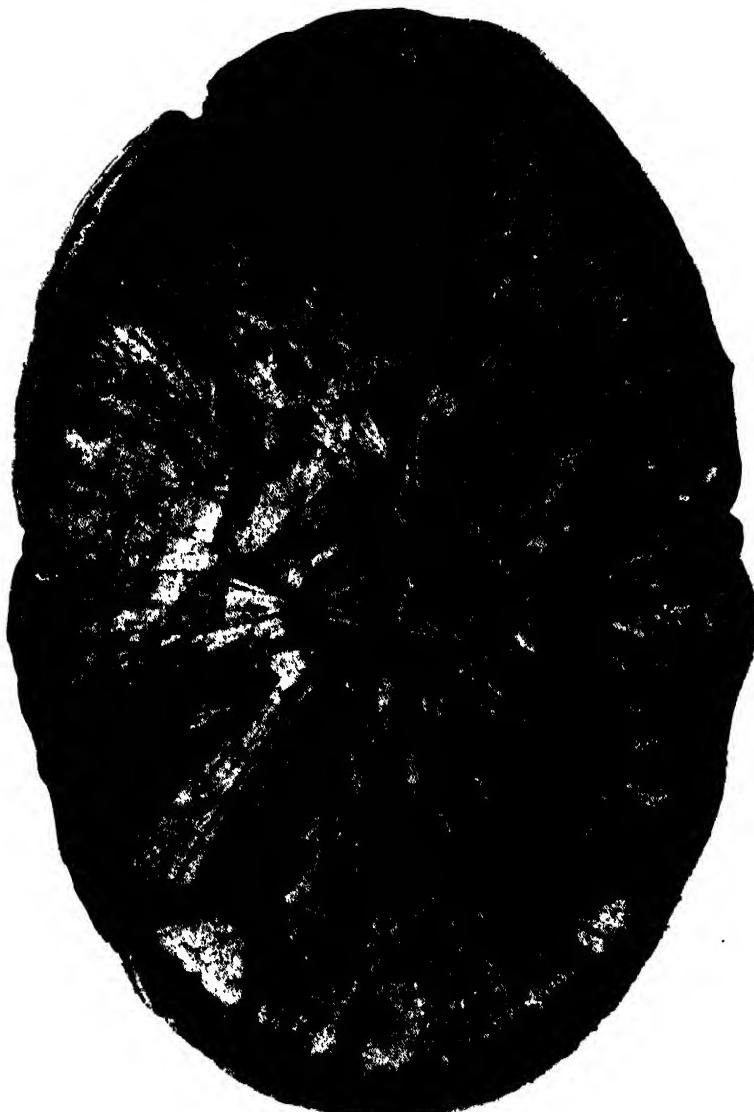
FIG. 8.—Large, homogeneous crystalline cholesterin gall-stone, after B. Naunyn.
(Enlarged 5x.)

the water a few large spheres produced in this way by guttulate separation. These spheres are at first myelin-like, transparent, soft bodies, which later, by the occurrence of crystallization with separation of fat, become radially-marked firm spheroliths.

If a gall-bladder filled with bile be tied off for a long time, the above-named conditions for guttulate separation of cholesterin are realized. Bile always contains, in addition to cholesterin, small quantities of fat dissolved in its cholate. Now in stasis of the bile, as the experience of the surgeon and the pathologist proves, the concentration of the cholate is gradually diminished by autolysis and resorption until finally a water-clear, almost cholate-free fluid is left; but the cholesterin remains in undiminished quantity and is ultimately in excess. The increasing impoverishment of the bile in cholate content, compels small quantities of cholesterin to separate out from time to time. But owing to the presence of fat it is guttulate separation which occurs, and since in such simple stasis, foreign substances are lacking, there is nothing to prevent the aggregation of the droplets.

In fact the method of formation of cholesterin in the bile under these conditions, corresponds exactly with that of the spheres in the experiment above. In the first stage we find these myelin-like, gelatinous droplets. These have been studied by B. Naunyn [6] in human bile and he has pertinently characterized them as the "precursor" of the gall-stone. As crystallization goes on they become firm, homogeneous, radially-marked spheroliths which, by

FIG. 9.　　　　　FIG 10.　　　　　FIG. 11.

Growth through drop-wise deposition of cholesterin on the surface of biliary calculi. The size of the separate spherules is very small in Fig. 9, larger in Fig. 10, largest in Fig. 11. (Natural size.)

continual addition of new droplets, reach a size of several centimeters in diameter. It is very remarkable that, entirely independently of physico-chemical investigation, anatomicopathological findings have demonstrated that the typical product in simple stasis of bile is the pure, homogeneous, radially-striated cholesterin stone (L. Aschoff and Bacmeister [7]). The process of development of this "pure cholesterin stone" may be illustrated by photographs.

Figure 6 shows the first stage of the still microscopically small, guttulate separation. In some drops (*a*) no structure is visible, in others (*b*), however, the beginning of the crystalline radial-striation is present (in the figure visible only at the dark kernel and isolated rays). Some droplets (*c*) show how they grow in size by the union of several, as they have 2, 3, and 4 centers of crystallization.[*]

The formations shown in Figure 6 were produced artificially from aqueous cholesterin-cholate solution, to which had been added a trace of fat (see above).

Figure 7 shows a natural cholesterin gall-stone of very small size and, so to speak, youthful state. Just as in the droplets of Figure 6, but here more clearly, we have in the center the radially-striated crystallization, though this is lacking at the periphery, which is made up of later additions.

And finally, Figure 8 shows a large, mature cholesterin gall-stone in which

[*] Naunyn, B., "Klinik der Cholelithiasis," F. C. W. Vogel, Leipzig, 1892.
[7] Aschoff-Bacmeister, "Cholelithiasis," Jena, 1910, also L Aschoff, *Münchener med. Wochenschr.*, p. 1750 (1913).
[*] The structures rimmed by sharp dark walls, are fat droplets which, at the beginning of the crystallization process separate out from the myelin-like balls of cholesterin.

—neglecting the thin superimposed outer stratum of different composition—the entire mass of the stone exhibits a homogeneous radially-striated spherolithic character. The structural origin of the pure cholesterin stone, as shown in transverse section, can be recognized in this series of illustrations without further description.

To show the growth by guttulate separation such pictures of the superficies of the stone as are given in Figures 9 to 11 are of value.

Figure 12 shows an unusual but quite definite example. On the surface of this very hard cholesterin stone, with well marked radial crystallization, appears (see the arrow in the figure) a large, gelatinous drop of myelin-like, transparent cholesterin, so characteristic, that there is here no doubt that this drop is, as a whole, an appendage to the stone.

FIG. 12. — Natural cholesterin gallstone with attachment of a large drop of still soft, fresh, g l a s s y cholesterin. After H. Schade. (Natural size.)

This method of growth of a stone by attachment of so large a cholesterin drop is, however, unquestionably very rare. The pellet-like structures on the surface of the stone shown in Figure 11, for instance, certainly did not originate as independent large drops floating free in the solution, but rather in this case certain definite points on the surface of the stone have served as centers of adsorption. At these points the cholesterin drops have settled while yet in the first stage of their formation, and, just as happens when they remain free in the solution, further growth by aggregation has proceeded to form larger spherical or hemispherical structures.

This kind of *appositional growth with the formation of humps or tubercles* on the surface of the stone, is apparently a universal characteristic of the development of the formations which arise in undisturbed guttiform separation. In the subsequent *completion of crystallization* the rod-like crystallization of the cholesterin (for the beginning of this see the middle of the stone in Figure 8) finally proceeds to the formation of these humpy marginal masses, and thus leads to a surface contour such as is shown in a somewhat schematic way in Figure 13.

Such development and such moulding of the separating cholesterin occurs in stasis of bile only when the fluid cavity is practically free from other deposits of crystalloid or colloid nature. Of course, strictly speaking, adsorbable foreign substances are always present in bile. It is therefore evident that the products here characterized as "pure cholesterin stones" are not absolutely free from adsorbed admixtures (mucus, albumin, calcium-bilirubin). After

FIG. 13.—Old true cholesterin stone. Surface appearance. After B. Naunyn. Ridge-like crystallization of the cholesterin continued to the surface. (Enlarged about 2x.)

dissolving the cholesterin by suitable methods, there will be found, even in the "purest" cholesterin stones, a trace, at least, of more or less "organic scaffolding" (Ebstein, Naunyn, and others). In stone formation this "organic scaffolding" is, however, to be considered as for the most part incidental.* The process here involved, that is, guttulate separation, with its secondary crystalline development, also takes place when the before-mentioned incidental impurities are positively excluded, i.e., in the sole presence of cholesterin in

* The form of the cholesterin crystals may easily undergo modification under the influence of adsorbed mucus, etc. (see below).

an artificial fatty cholate solution. Essentially the process remains unchanged even in the presence of small quantities of incidental impurities. In the presence of larger quantities of such impurities the cholesterin drops soon become thicker and firmer because of adsorption films, with the result that the stone, as the "scaffold substance" increases in thickness, loses more and more the uniform development of its radial striations. This disturbance may easily extend so far that the adsorption films prevent the fusion of the drops into a uniform crystalline mass. The structures formed under these circumstances will be considered below.

Guttulate separation may occur also in the urine. Here likewise the investigations of H. Schade have afforded an insight into the physico-chemical laws. Under certain conditions uric acid in urine may play a rôle similar to that of cholesterin in bile, so that, by guttulate separation, so called "pure uric acid stones" occur. We have already seen above that for stabilization of uric acid- or urate- supersaturation, the presence of an intermediary colloid (it may here be designated briefly as a "U-colloid") is of great importance. A reaction of the solution between the H-ion concentrations of 10^{-7} and $10^{-5.5}$, that is to say, a slightly acid reaction, gives a very sharply marked and steep optimum for the separation of these colloids.

Figure 14 shows side-by-side, under varied reaction, the curve of pure solubility, the curve of colloid stabilization, and in the third place particularly, the curve of "holding in solution."

Neutral

Alkaline ← → Acid

— — — Curve of normal solubility
.... Curve of colloid stabilization
——— Curve of "holding in solution"
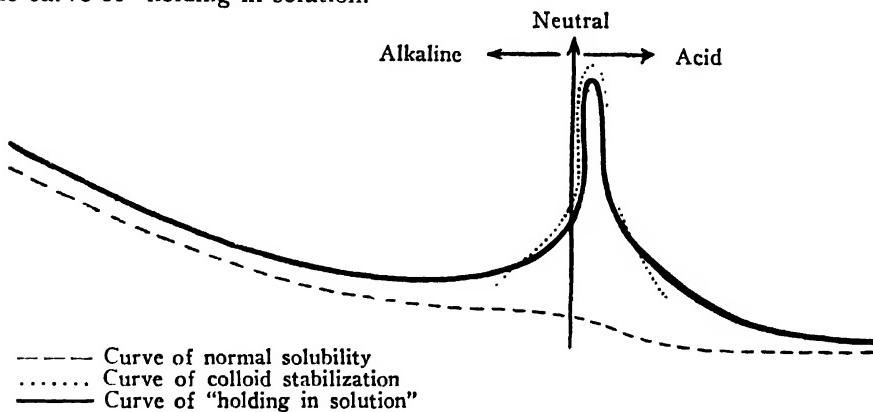
FIG. 14

As may be noted, the curve of abnormal retention of supersaturation ("holding in solution") coincides with the curve of stabilization of the intermediate U-colloids. In the narrow range of reaction wherein the U-colloid, in consequence of its peculiar stabilization, evidently and demonstrably appears, the solution has a zone of abnormal freedom from precipitation. Moreover this appearance of the U-colloid can be seen even in the precipitate. The limits of reaction between the H-ion concentrations of 10^{-7} and $10^{-5.5}$ determine for precipitation from U-supersaturation a *zone of strongly marked guttulate separation* and, likewise, continuing into the final stage of the separation, a *zone of appearance of crystalline spheroliths*.[8]

[8] A detailed investigation of these conditions is reported by H. Schade, *Z. klin. Med.* 93, 1-65 (1922). "The physico chemical laws of uric acid colloids and super-saturated uric acid solutions."
The mechanism of guttulate separation appears to be this: ultramicroscopic aggregates (crystalline

The following table illustrates, by the example of a particular experiment, such dependence of sediment formation on the reaction of the medium.

Mode of Precipitation of an 0.8 per Cent Sodium Urate Solution (20°) According to Varying Reactions of the Medium.[*]

In 4 cc. of Sol'n	+ 0.2 ⅒ NaOH	+ 0.5 ⅒ NaOH	+ 0.2 ⅒ NaOH	Alone	N-zone		+ 0.1 ⅒ HCl	+ 0.2 ⅒ HCl	+ 0.5 ⅒ HCl	+ 1.0 ⅒ HCl
					+ 0.2 cm. ⅟₁₀₀ HCl	+ 0.4 cm. ⅟₁₀₀ HCl				
	I	II	III	IV	V	VI	VII	VIII	IX	X
Precipitate after 20 hours.	Pure urate crystals in regular formation.	Tuft-like crystals. In part, large double tufts.	Radiating crystals in balls.	Spherical masses with protruding crystal radiation.	Pure, quite clear, fluid spherules with crystal-free contents.		Spherical masses with crystalline granules.	Ball-like aggregations of small crystals.	Small crystalline granules. In part, spherical clumping.	Pure uric acid crystals of typical shape.

Figure 15 shows the difference between the precipitates of I, V, and X in photomicrographs of the same magnification.

With simple technique [11] it is possible to make, on a microscopic slide, U-droplets as large as about 40 μ, i.e., nearly five times the diameter of a red blood corpuscle.

For the study of the more immediate antecedents of guttulate separation, these preparations are peculiarly adapted. At first the U-droplets are clear, sharply outlined, and of light yellowish color, not unlike oil-droplets; they can be distorted by pressure, they are always bounded by curved lines, and on pressure angles are never seen. Their fluid character is therefore demonstrated. Their fluidity is further shown by the manner in which these droplets coalesce to form larger globules, as may easily be seen under the microscope. Figure 16 sketches such growth of the droplets.

While this growth is going on the original glass-clear appearance of the globule is lost; it seems like a molten mass, partially cooled, slowly becoming more and more firm and crystalline. But in all these fresh U-droplets, while they are still, under the microscope, entirely clear and undoubtedly fluid, we can already see a regular stratification of the molecules; like "fluid crystals"

(r not) form at many crystallization centers, and as their kinetic activity decreases with their increase in mass, surface forces bind them into spherulites. The spherulites may later form dendrites, and these orient into crystals, on which the ultramicroscope may reveal particles which could find no room in the space lattice. These intermediate steps are quite common. See J. Alexander, "First Colloid Symposium Monograph," 1923, and papers by Kruyt, Endres, and Alexander, Vol. I of this series. In the Trent process of coal recovery, the oil binds the coal dust into spherical particles called "caviar." *J. A.*
[*] Taken from H. Schade, *Z. klin. Med.*, **93**, 35 (1922).
[11] For details see H. Schade, *idem.*, p. 5.
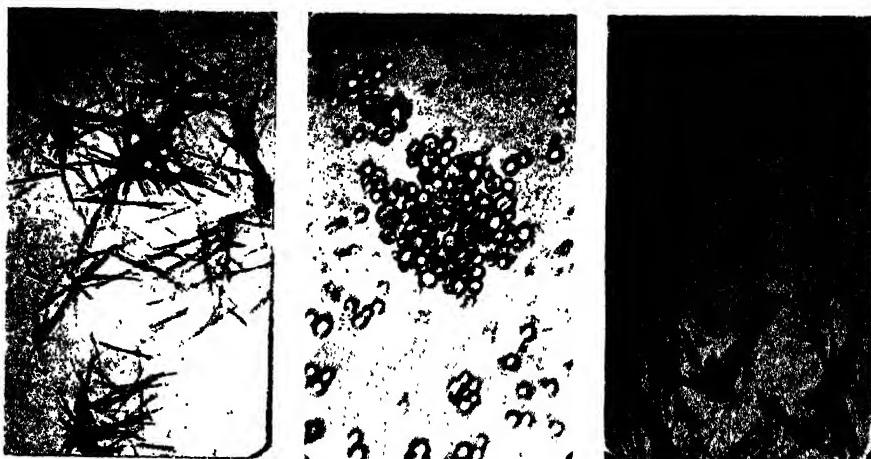
| Alkaline reaction: | Zone of colloid protection. | Acid reaction: |
| Urate crystals. | Guttulate separation. | Uric acid crystals. |

FIG. 15.—The guttulate separation as intermediate phase between the urate and the uric acid precipitation. After H. Schade.[11]

so also these droplets, when examined with crossed Nicols, show in a most beautiful way the polarization cross. (Fig. 17.)

When, however, under ordinary illumination the turbidity of the drops begins to appear, observation by polarized light shows that the cross fades and presently entirely disappears. If we crush the balls just as they are becoming turbid, we see upon the broken surface facets with sharp angles and even entire crystals, evidences that the mass has already become firm and crystalline. From U-supersaturation it is possible in the same experiment, by slower precipitation, easily to obtain balls up to about 2 to 3 mm. in diameter, and on again bringing into a supersaturated condition we may obtain instead, a further growth by apposition.

Figure 18 shows the chief stages of the origin of a spherolith structure in a "pure uric-acid stone" from precedent guttulate separation.

FIG. 16.—Growth of the U-droplets as observed under the microscope. After H. Schade.

The rôle of the "organic scaffolding" in these stones is, like that in the "pure cholesterin stones," usually only accidental, for here also stone-formation may take place without any scaffolding at all, out of pure watery supersaturation. It is important to call special attention to the fact that, in entire accord with their herein-described genesis, anatomico-pathological observations also support the view that the "pure uric-acid stones" are formations from simple supersaturation without co-existing inflammation. (See below.) (L. Aschoff and O. Kleinschmidt.[12])

[11] For details regarding this zone of guttulate separation see H. Schade, idem., pp. 38 and 47-53.
[12] Kleinschmidt, O., "Die Harnsteine," with a foreword by L. Aschoff, J. Springer, Berlin, 1911.

Both kinds of "pure crystalloid stones," the cholesterin and the uric acid stone, have physico-chemically a closely related origin; both owe to guttulate separation their beginning, their increase, and their further growth into a homogeneous mass, and in both alike the ground plan of their crystallization, their uniform radially-striated structure results as a consequence of drop-wise separation. Since uric acid has less tendency to exist in long needle- or beam-shaped crystals, therefore uric acid stones, particularly the larger specimens, show less well-marked radial striation. One of the most common forms in which they occur in the branching pelvis of the kidney, is shown in Figure 19. The structure of such a stone is generally apparently homogeneous throughout its whole mass, the uniform, centrally-oriented radial striation is usually only barely suggested; since growth proceeds continually, i.e., without pause, layering is apt to be absent. Particularly to be observed in Figure 19 is the prominence of numerous nodules on the surface of the stone; these little lumps are here, as in the
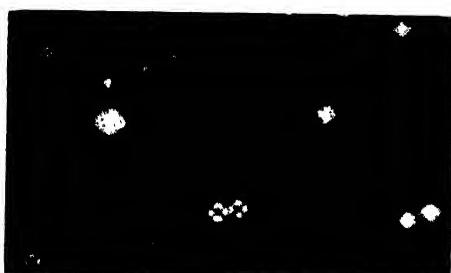
FIG. 17.—U-droplets in polarized light. After H. Schade.

cholesterin stone, additional evidence of an origin from guttulate separation. The occurrence of such precipitation and stone formation through guttulate separation in the urine, is, so far, proved for uric acid only. It is entirely probable that other crystalloid supersaturations show under certain conditions a like predominance of guttulate separation. Several observations regarding calcium oxalate especially point in this direction.

The *combined colloid-crystalloid stones* constitute the most numerous group

Greatly enlarged.
Stage of pure guttulate separation.

Greatly enlarged.
Stage of microscopical spherolith structure.

Natural size.
Stage of completed radially striated "pure uric-acid stone."

FIG. 18.

of the concrements of the human body. Here colloids (mainly albumin, arising from inflammatory processes) in association with crystalloids are not only more abundantly represented quantitatively, but the colloids have a marked influence upon the architecture of the stone, since by tangential invasion of the crystalloid radial-striation, they cause concentric layering. It is characteristic of the combined colloid-crystalloid stone that in it concentric layering and radial striation occur in combination. And it depends upon the relative quantity as well as upon the formative force of the one as compared with

the other constituent, whether concentric layering or radial-striation pre-
dominates. Figures 20 to 22 show this:

On the greater quantity of colloid in
these stones depends the "organic scaffold-
ing" which is always well marked. But, it
is not the excellent formation of the scaffold-
ing,[13] nor the addition of the colloid-con-
ditioned layering * to the radial striation
which makes the differentiation of these
stones from the pure crystalloid stones of
such practical importance. The chief differ-
ence lies in something entirely different in
the mode of origin. The pure crystalloid
stones, judging by their constitution, are
supposedly formed in such a manner that
the precipitation of the crystalloids from
solution precedes a distinct drop-wise separa-
tion. Now this supposition is fulfilled by
only a certain few crystalloids and by those
only under certain conditions. The com-
bined crystalloid-colloid stones, on the other
hand, may be formed in either way from the

Fig. 19.—Typical true crystalloid
stone. Uric acid stone without
layering of the interior, also with
clear evidence of dropwise addi-
tion.

same crystalloid material, because here the colloid plays the rôle of uniting the
mass firmly and uniformly into a concrement. H. Schade [14] was able artificially

Fig. 20. — Choles-
terin - c o l l o i d
stone. (Transpar-
ent section. Na-
tural size.) Type
·o f predominant
radial striation.
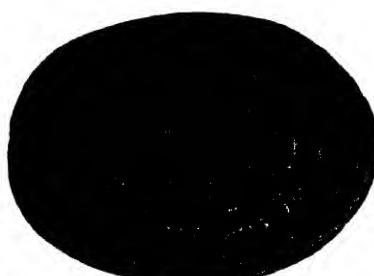
Fig. 21.—Uric acid colloid stone.
(Cross section. Natural size.)
Type of the equal influence of
concentric layering and radial
striation.
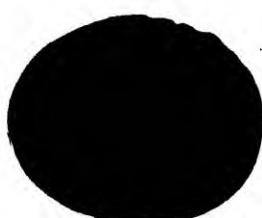
Fig. 22.—Bilirubin-calcium
colloid stone. (Cross sec-
tion. Natural s i z e.)
Type of predominant
concentric layering.

COMBINED COLLOID-CRYSTALLOID STONES.

to produce stones of this type by allowing coagulation of fibrin to occur in
solutions which contained freshly precipitated, sediment-like, discrete crystal-

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[13] For details concerning the structure of the stone-scaffolding see H. Schade, *Münchener med.
Wochenschr.,* Nos. 1 and 2 (1909).

* In addition to the layering due to the colloids, other layerings are possible and frequent from
other sources, especially from alterations in the chemical composition of precipitated materials, and
from purely time influences, in so far as the deposit occurs in portions, with longer or shorter pauses
between, whereby the structure of the older layers, changed through secondary processes (see below),
may be distinguished from the new ones.

[14] *Münchener med. Wochenschr.,* Nos. 1 and 2 (1909).

loid material. By no means all, but only the irreversibly precipitating hydrophilic colloids have this action; the fibrin in the experiments just alluded to is an example of such. Recognition of this law clarifies markedly our understanding of the mode of stone formation in the urine and in the bile. Crystalloid precipitation in normal urine and in normal bile because of the absence

of an accompanying separation of irreversible colloid, cannot possibly result in stone formation. Only when, through morbid processes, irreversibly separating colloids, such as fibrin or other protein, are present in urine or bile, can transformation of the loose crystalloid sediment to a stone take place. With this physico-chemical result accords well the clinical experience that the appearance of the combined colloid-crystalloid stone always is accompanied by an inflammatory process, with the separation of irreversible albuminous colloid. It is especially important practically to note that in the experiment already alluded to very minute quantities of irreversible colloid (e.g., 0.07% fibrin, Schade [16]) were found sufficient to transform the process of sediment-formation into that of stone-formation. If the process of stone-formation goes on very slowly, it occasionally happens that the separation of colloid and also of crystalloid is so small as to elude observation entirely, in

Fig. 23.—Special type of combined colloid-crystalloid gallstone: *Stratified stone* = "common gall-stone." (Sketch of transverse section.)

spite of noticeable growth of the stone. And so, in the urine for example, in the presence of a very slight inflammation, stone-formation or growth can occur in the absence of noticeable precipitation. In all these stone-formations the relative concentration of colloid and crystalloid in the solution may be
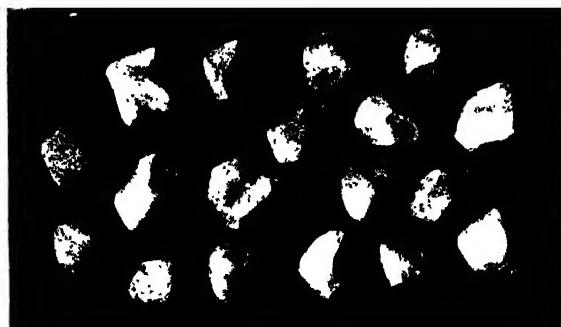
Fig. 24.—Typical external forms of "common gall-stones" (stratified stones.) These are artefacts, made by subjecting plasticin spheres to elastic wall pressure in a rubber finger-cot (after H. Schade).

quite unlike that which is found in the stone; on the contrary, in consequence of adsorptive action decided shifting of quantity relationships of the separate materials is the rule.*

The experiments give very helpful information towards a partial solution of question as to the part played by the colloid in the formation of these

[16] *Münchener med. Wochenschr.*, Nos 1 and 2 (1909).
* See another example below under the Carlsbad pea-stones (Part V).

stones. If the crystalloid constituents of a stone are dissolved by acid or other appropriate solvent, there remains a firm coherent skeleton which shows exactly all details of the structure of the stone. If, however, the albuminous skeleton is dissolved, e.g., by antiformin, the entire crystalloid content is dissipated, and nothing remains but a slimy mass of finely divided crystals (Schade).[16]

Another kind of the combined colloid-crystalloid stones are the *layered stones* (Schalensteine) which (as indicated by the name generally given them —"common gall-stone") are the concrements most often found in the gall-bladder. They are never found singly, but always in larger or smaller groups, sometimes of 100 and more, in the gall-bladder. Figures 23 and 24 show their usual structure. In these stones the kernel and the layers may always be distinguished. As a rule the kernel consists of a mass predominantly composed of albumin and calcium bilirubin, showing a structure like that in Figure 22. The layers are also built up of a mixture of colloids and crystalloids; but here the form of the structure is dominated by cholesterin, which in these stones determines layer structure.

Freshly precipitated cholesterin is highly plastic, this property being favored by small amounts of fats; it is easily moulded, like snow, only more so. It is especially adapted to the formation of firm thin layers (as thin as paper, and less). This plasticity may be demonstrated by the following simple experiment of H. Schade: A hot alcoholic solution of cholesterin (e.g., 0.2 gram cholesterin in 10 cc. of 96% alcohol) is allowed to cool slowly to form tufts of soft needles in a test tube. If a small cup of wire mesh is now forced down through the tube, and then withdrawn, a coherent layer of cholesterin remains (Fig. 25). Even with extremely dilute solutions, such layers may be removed and spontaneously allowed to dry to coherent films.

The smallest amounts of irreversibly precipitated albumin included, make the layers ever so much stronger. They look like silk, and often even like mother-of-pearl, because of the presence of fine layers of crystals in plane-parallel formation. These resemble the layers in common gall-stones. The silky cholesterin is usually stained to brilliant and

Fig. 25
Experiment showing stratification of cholesterin (after H. Schade).

beautiful shades, by adsorption of coloring matters like bilirubin, biliverdin, bilicyanin, bilifuscin, etc. Such stones are frequently not unlike pearls.

The slightest pressure suffices to make layers of the fresh cholesterin. Therefore, when there are many stones in the gall-bladder, so that they rub against each other, the layered structure is the rule; it is not found where only one stone is present. The reason layered gall-stones occur so often is that gall-bladder inflammation, a common clinical symptom, develops exactly the conditions for their formation. Pathologically altered masses of albumin, developed by the inflammation, carry with them, on precipitation, calcium bilirubin;[17] thus is formed the nuclei of "common gall-stones."

Continued stasis of the bile results from swelling and occlusion of the

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[16] *Med Klinik*, No. 15, 1911.
[17] See B. Naunyn, "Klinik der Cholelithiasis," Leipzig, pp. 18-19, 1892.

gall duct, consequent on inflammation; this forces a period of prolonged albumin-calcium bilirubin separation.* Because of the presence of the albumin-calcium bilirubin nuclei, the cholesterin can not unite into a solitary stone; it is layered about these nuclei.** As the stones grow, and the gall-bladder cavity becomes filled with them, they press against each other and form layered, rather than radiating, masses of cholesterin. Just as isolated stones of radiating structure constitute the typical end-product where there is stasis of bile without inflammation, so with almost the same regularity where inflammation is a precursor of the stasis multiple layered cholesterin or "common" gall-stones are formed on albuminous nuclei.

In rare cases, where the inflammation permits the formation of few albumin-calcium bilirubin fragments, the subsequent stasis is followed by only a few (say 2 or 3) cholesterin stones, which, because of lack of mutual pressure, permit the radial formation of cholesterin to establish itself. One and the same stone may show two kinds of layers (see Fig. 26), differentiated

a   b

FIG. 26.—Diagram showing layering of cholesterin crystals in scaley (a) and in layered structure (b) (after H. Schade).

by the direction of their crystallization. When pressure has been lacking the layers are as indicated in b; where pressure has operated, as in a.

A second important anomalous type of combined colloid-crystalloid stones are the so-called *"tube casts,"* which are rare in humans but common in the inflamed gall duct in cases of liver fluke in cattle. Figure 27 gives photographs of typical cases of such concretions. Although these "tube casts" appear different from the stones heretofore described, both are formed from a simultaneous precipitation of colloids and crystalloids and often have a concentric layered structure. In fact, under the same general conditions, a supersaturated solution may deposit its precipitate at a given place either as a wall-like compact or as discrete particles. Surface tension is the main factor controlling which form the precipitate will take. Under normal conditions the surface tension between the mucous membrane and the physiological fluid bathing it, is practically zero: therefore healthy mucous membrane never shows a surface skin and is never covered by a layer of precipitate.

Because of this property of the mucous surfaces, which is indispensable to its physiological cleanness, precipitation phenomena in the body cavities proceed quite differently from test-tube experiments, acting as though no extraneous surfaces were present. In the absence of such outside influences, fluids in body cavities having healthy mucous membranes, exhibit in very perfect form all kinds of separations (guttulate precipitation, colloidal precipitation, etc.).

Quite different is the case with diseased mucous membrane, where profound colloidal changes in the cells may determine a high surface tension between the membrane and the fluid, which makes the membrane figure as an

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* This is determined by the bile losing cholates by resorption and autolysis. The same is the case in the formation of solitary cholesterin stones. Bacterial decomposition of cholates produces a similar effect.

** Small amounts of albumin and calcium bilirubin are included.

adsorbing surface. The more its adsorbing influence dominates as a consequence of illness, the stronger will be the wall-like deposit of the precipitate. This establishes one of the conditions under which are formed coherent

½ natural size

Stoney hard tube casts, completely formed.

Lower lying portion of the gall-bladder, with coherent deposit forming a marked "incrustation" of protein.

Two liver flukes which caused the disease.

natural size
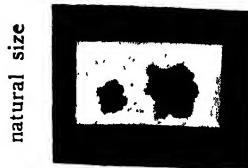
Typical bilirubin - calcium - protein stone, detached variety, taken from the gall-duct of the same animal.

Fig. 27.—Gall-stones from liver-fluke disease in cattle (after H. Schade).

adhering concretions or "tube-casts"—the formation of free concretions may even occur simultaneously.

Not infrequently other intercurrent processes are competitors here. Thus the diseased membrane may produce abnormal, coagulating albuminous secretions, which become hard as stone because of secondary depositions of crys-

talloids. Such a process is known as *"incrustation,"* and is to be distinguished from the concrement formation here described (see below).

We have now considered the chief characteristic of the main varieties of urinary and gall-stones, namely purely colloid stones, purely crystalloid stones, and the combined colloid-crystalloid stones (both types). Transition forms and deviations from these types are by no means uncommon. Apart from divergences due to incidental differences in the crystalloid and colloid materials, individual stones often show a *change in type from one layer to another,* consequent on a reversion of conditions in the external milieu during formation of the stone. One example has been given above, in speaking of layered gall-stones. Besides referring to Figure 33, there is here shown Figure 28 which exhibits a marked instance of transition in type. In addition to the sharp differentiation of the layers, this stone is important because the history of the case and the evidence found on operation confirm the changes in conditions of formation as deduced from the structure of the stone itself: first, a period of simple stasis without inflammation, leading to the formation of a simple radial cholesterin stone as a core; then a prolonged period of stasis with slight inflammation, leading to formation of radial-concentric layers; and thirdly, a month of high inflammation with extensive precipitation of albumin and calcium bilirubin, forming the final layer which still shows concentric form (H. Schade).

This case shows how the above principles may give us an insight into the origin of a stone. Instead of considering gall and urinary stones as enigmatical structures, the physician may consider them as records left by nature of the development of the disease. It is to be hoped that this advance may lead to a rational basis for treatment of stone.[18]

FIG. 28. –Gall-stone showing different modes of deposition (after H. Schade). Natural size

The explanation we now have of gall and urinary stones is entirely physicochemical. Colloid chemistry has proven most important. It is interesting to trace how far back physicians have thought that colloids are concerned in concrement formation. Even in the classic age the fathers of medicine, Hippocrates and Galen, with astounding foresight, attributed the formation of these stones to an accumulation of mucus, which clung to the organ and permitted the stone to form. The first experimental observation of colloid nature was that of A. von Heyde, who in 1684 demonstrated a residual "framework" on dissolving out the crystalloids from urinary calculi. Further information was given in the book on "Mikrogeologie" by Meckel von Hemsbach, published in 1856 by Th. Billroth after the death of the author. One paragraph may be quoted to show what importance this author ascribed to the colloidal constituent in stone formation.

"Two basic factors underlie the formation of every true gall or urinary stone; first, the presence of an organic substance, mucus, in which there may be a deposition of salts; second, a suitable urinary or gall fluid to serve as the mother-liquor for these sediments. The decomposable organic substance,

[18] See H. Schade, *Med. Klinik*, No. 15 (1911).

mucus, is unquestionably necessary, because urinary salts and gall substances, of themselves, can yield only crystalline, pulverulent, or granular precipitates, and never large pieces. Stones are formed only when an organic binder is carried down too."

W. Ebstein,[19] in 1884, realized that mucus is unsuitable for formation of stones: he recognized a specific frame-forming substance which "petrified" itself with the stone and is lacking in normal urine, as essential to stone formation.

Subsequently Moritz,[20] Pfeiffer,[21] Posner,[22] and Schreiber[23] showed that even crystals deposited from normal urine had an organic framework; and the idea of such a framework became better established. In 1892 Naunyn[24] made a basic investigation of gall-stones from a clinical point of view. Here, for the first time the soft myelin-like balls of cholesterin found in the gall bladder, are regarded as the "precursors of gall-stones." No explanation was given as to the formation of gall-stones; on classical grounds, Naunyn attributed this to inflammation. The physicochemical attack on the problem began, in 1909, with the work of H. Schade.[25] Practically at the same time L. Aschoff and his pupils Bacmeister and Kleinschmidt, from the anatomical-physiological point of view, reached the important conclusion that in both gall and in urine we must differentiate between stones arising without inflammation and stones consequent on inflammation.[26]

II. OTHER CONCRETIONS OF THE HUMAN BODY, WITH A RÉSUMÉ OF THE GENERAL LAWS OF CALCULUS FORMATION IN MAN

Just as in the bile and in the urine, so also in the remaining fluids of the human body, concretions are, under morbid conditions, very frequently found. It is noteworthy that these formations show the same structures as were described in the previous section as typical for biliary and urinary calculi, in spite of the differences which exist in the chemical nature of the materials from which they are formed. In the first place, mention must here be made of *intestinal* stones, stones of the *pancreas* and of the *salivary glands, prostatic* stones, *lime concrements* in old softened tissues, the *corpora amylacea,* "*rice bodies*" of the joints, *thrombi* of the blood, and finally "*brain sand.*" Among the several examples of these various formations are represented all three types of the above-described concrement forms. Some of the "rice bodies" of the joints and especially some of the thrombi of the blood, show excellently the simple concentric stratification of "pure colloid stones." In the example of a prostatic concretion (see the fragment pictured in the upper part of Fig. 29) the recurrence of a pure crystalline radiation may be demonstrated. By far the most frequent are the concretions due to combined crystalloid-

[19] "Die Natur und Behandlung der Harnsteine," Wiesbaden, 1884.

[20] Moritz, "Ueber den Einfluss von organischer Substanz in den Kristallinischen Sedimenten des Harns," 14th Congress for Internal Medicine, Wiesbaden, 1896.

[21] Pfeiffer, E., Aetiologie und Therapie der Harnsauren Steine," 5th Congress for Internal Medicine, Wiesbaden, 1886.

[22] *Z. klin. Med.,* 5 (1885), and 16 (1889).

[23] Virchow, *Archiv.,* 153, 147 (1898).

[24] Naunyn, B., "Klinik der Cholelithiasis." Leipzig, 1892

[25] For résumé, see H. Schade, "Die physikalische Chemie in der inneren Medicin," Leipzig-Dresden, 1920.

[26] Aschoff-Bacmeister, "Cholelithiasis," Jena, 1910; and Kleinschmidt, O., "Die Harnsteine," Berlin, 1911. One other preliminary piece of work in this direction is important. J. Boysen, "Über die struktur und Pathogenose der Gallensteine" (with preface by Prof. Rousing of Copenhagen), Berlin, 1909.

structure, while crystalloids favor a radial striation. Where large percentages of both constituents appear, both types of structure co-exist, interwoven.

In humans, concretions grow by apposition. Any individual layer maintains for a long period the constitution and structure reflecting the properties of the fluid at the time of its formation.

The old question as to the *significance of nuclei in stone-formation,* is still much mooted in the medical literature, and is closely related to the above. It is wrong to rule out a "nucleus," even though any one of the three types of stone may be formed without it. A portion of the precipitate, which has preferential development in point of time or otherwise, may function just as effectively as a foreign nucleus; so there is no essential difference between "nucleus" and the balance of the stone. How is it, then, that relatively often we find as nuclei what are unquestionably foreign bodies (e.g., tissue fragments, worm eggs, even simple air bubbles)?

The explanation is easy. Whatever produces in the urinary or gall bladder an area of marked surface tension, breaks down the physiological protection against stone-formation; and adsorption makes this area an accumulator of whatever mass may precipitate. Generally, however, another factor appears: the foreign body produces inflammation of the mucous membrane, and by causing exudation of colloid into the cavity, supplies another very important means of stone-formation. The stone itself, in all stages of its formation, is in effect a foreign body, so long as it shows interfacial tension against the solution. It is, however, possible that a stone may acquire such surface properties that adsorption is practically lacking; such stones may remain for decades within the body cavity without causing their carriers any trouble.

Not infrequently in the medical literature, the encasing of foreign bodies with stone-material is considered as "simple incrustation" in contradistinction to "true concrement formation." We have seen, though, that there is no real difference. This is not the case with *incrustation of the mucous membrane,* which, as pointed out above, is essentially a different process—an exudation of albumin which gels before it gets free from the mucous membrane, forming a preliminary crystalloid-free coat. In this coat, crystalloids, especially calcium salts, may be gradually deposited by the most varied causes, e.g., chemical combination, adsorption, perhaps even diminution of colloidal protection for the supersaturated solution in the neighborhood of this precipitated albumin. Here the albumin has never been free in the cavity, and its association with crystalloids is secondary; the term "incrustation" is justly applicable. Under special conditions formations of this type may occur in the human body, e.g., transformation of a dead foetus into "lithopaedion." They lack the typical structure of a concrement. But in rare cases, owing to mechanical or other disturbances, atypical stones of highly irregular structure may arise, which renders classification difficult. L. Lichtwitz ("Ueber die Bildung der Harnund Gallensteine," Berlin, 1914) takes the view that concrement formation is an incrustation process, in which colloid precipitation is the initial step, followed by deposition of crystalloid material.

Crystal form is materially influenced by the properties of medium existing at time of formation. Lyophile colloids exert a powerful influence. Thus R. Marc [27] found that uric acid, which (see Fig. 15) forms the well-known

[27] Marc, R., "Ueber Kristallization aus wasserigen Lösungen," *Z. physik. Chem.,* 61, 385; 67, 470; 68, 104; 73, 685; 75, 710, and 79, 71.

* Attention must be here directed to the important but much neglected work of Dr. William Miller

whetstone shaped crystals from aqueous solution, gives striped double pyramids in the presence of methylene blue, but guttulate, thin-skinned crystals in the presence of Bismarck brown. Long ago physicians had made observations of this character. In 1879, Ord collected his observations in a monograph, "The Influence of Colloids on Crystalline Form and Cohesion" (London). In 1882, Ultzmann [28] found that stone-forming substances crystallize quite differently in concretions than when free in the urine. Posner [29] confirmed these observations, and extended them to calcium oxalate: in con-

<div align="center">
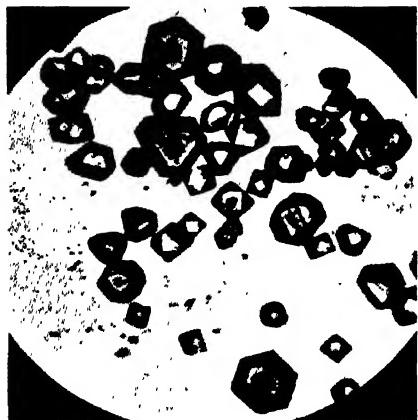
Fig. 31a.   Fig. 31b.

</div>

Fig. 31.—Influence of methylene blue (as an impurity) on the mode of crystallization of silver chloride.

(a) Unaffected regular crystals.
(b) One-sided growth of crystals consequent on inhibition of crystal growth due to adsorption of the impurity at the other faces (after H. Freundlich).

cretions are found neither the envelope or dumbbell forms, but instead, fine needles, which exhibit strong double refraction. Sabbatini and Selvioli [30] showed that in the presence of colloids, calcium carbonate also shows anomalous forms. The systematic investigations of R. Marc (*loc. cit.*) laid the basis for understanding the appearance of different crystalline forms. As H. Freundlich in his "Kapillarchemie" explains in detail,[31] the presence of an adsorbable foreign substance generally favors a lopsided crystal growth. He uses crystals of AgCl as an example (see Figs. 31, *a* and *b*) : *a* shows the structure of the uninfluenced crystal; *b*, the dendritic structure formed in presence of methylene blue. The various crystal faces adsorb differing

Ord, mentioned below, whose results, including reference to his predecessor and co-workers, are summed up in his book "On the Influence of Colloids upon Crystalline Form and Cohesion, with Observations on the Structure and Mode of Formation of Urinary and other Calculi," London, 1879; see "Science," 61, 184 (1925). Guttulate separation of cholesterin was observed and demonstrated by Ord and many principles of medical and biological interest discussed and shown in beautiful drawings. Proceedings of the Royal Society for 1879 also contain a paper by Ord, and other papers are in the literature of that period. *J. A.*

[28] "Die Harnkonkretionen des Menschen und die Ursache ihrer Entstehung," Vienna, 1882.
[29] *Z. Klin. Med.*, 9 and 16 (1885 and 1889); also Wilner, *Klin. Wochenschr.*, 1911.
[30] *Arch. ital. Biol.*, 58, 252 (1913).
[31] Freundlich, H., "Kapillarchemie," 2nd ed., Leipzig, 1922.

amounts of the foreign material, and are differently inhibited. Probably those crystal faces which grow most rapidly in pure solution, and therefore

FIG. 32.—Cholate stone from the urinary bladder, showing coral-like form (after E. Ziegler). Natural size.

have the strongest residual valencies, are the ones which adsorb most powerfully, being thereby more highly inhibited in growth. This explains the more or less complete change in crystal form, often along a line comparatively unimportant in an ordinary crystal.

Colloids also exert an important influence in leading to intermediate guttulate separation. Cholesterin and uric acid may even do this without the presence of a foreign colloid.* But colloids may play a rôle with other crystalloids. Thus Ord (*loc. cit.*) saw oxalates form radially-striated balls in the presence of gelatin. E. Hatschek [32] confirmed and extended these findings, using 5-20 per cent gelatin, and 1-5 per cent agar. According to H. Schade,[33] when calcium oxalate separates out in blood serum, it goes through guttulate separation. Similar observations exist with regard to calcium carbonate. Further experimental investigation will, we hope, explain the conditions under which form in urine the coral-like and bone-like stones like those illustrated in Figures 32 and 33.

FIG. 33.—Stratified colloid-crystalloid stone of the urinary bladder, showing bone-like structure of calcium phosphate and carbonate (after H. Schade). Natural size.

Let us now consider briefly the *secondary processes* occurring in the already formed stone. The forces of crystallization and of diffusion tend

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* This seems to be due to *auto-protection.* See Vol. I, this series, article on Colloidal Protection by J. Alexander. *J. A.*
[32] *Kolloid-Z.,* 8, 193 (1911).
[33] H. Schade, "Die physikalische Chemie in der inneren Medicin," 3rd ed., Leipzig-Dresden, p. 203.

to transform the relatively unstable mass into a stable crystal. A capital example is given by cholesterin gall-stones, whose transformation processes we have already considered. Another example is known as the cholesterin solitaire. Its crystalline development does not cease with penetration of the rays all over its periphery, as shown in Figures 8 and 13. The "devouring" of the small, unstable crystals by the big ones, and growth towards stable crystal form, proceeds steadily. A few of the older crystals on the
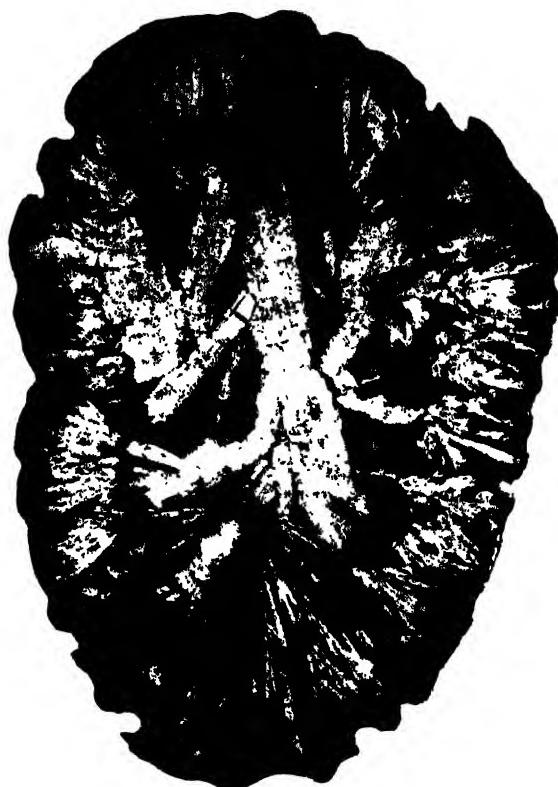
Fig. 34.—Solitary cholesterin stone showing thick rod-like crystalline transformation in the middle of a stone, with loss of the original structure (after Naunyn). (Enlarged 5x.)

interior gain dominance, and the original center loses its dominance over radiation. Such a secondarily modified stone is shown in Figure 34: nothing remains of the original finely crystalline structure which certainly must have existed in the middle. Instead, large crystals lie scattered in a way consonant with competition in mutual absorption. Only the rim shows the radial striations originally present, and 'even these are grouped into thick rods.

Ordinary gall-stones with concentrically layered cholesterin, not infrequently are also modified by slow crystallization of the cholesterin. Two

cross sections of one and the same stone (Figs. 35 and 36, from J. Boysen [34]), taken at different heights, show splendidly the breakdown of the exterior
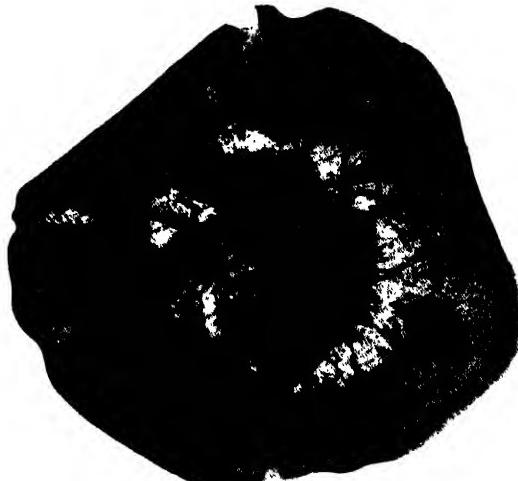
FIG. 35.

FIG. 36.

FIGS. 35 and 36.—Gradual radial transformation of cholesterin, in "common gall-stone" showing invasion of the structure by the banded layers (after J. Boysen). (Enlarged about 20x.)

layer by the transforming cholesterin. Cholesterin excels in crystallization capacity the other stone-forming substances found in humans, so that in other stones secondary transformations are rarely found.

[34] "Ueber die Struktur und Pathogenese der Gallensteine," Berlin, 1909.   Boysen attributed a different significance to these pictures.

The colloid portion of the stone may also change with time, often shrinking and making radial rifts leading outward from the middle, leaving an irregular hollow there, filled with fluid. Stones with much colloid content may even become friable because of colloid shrinking, and fall to pieces. Other influences may also cause gradual changes of form; thus multiple stones often

a   b
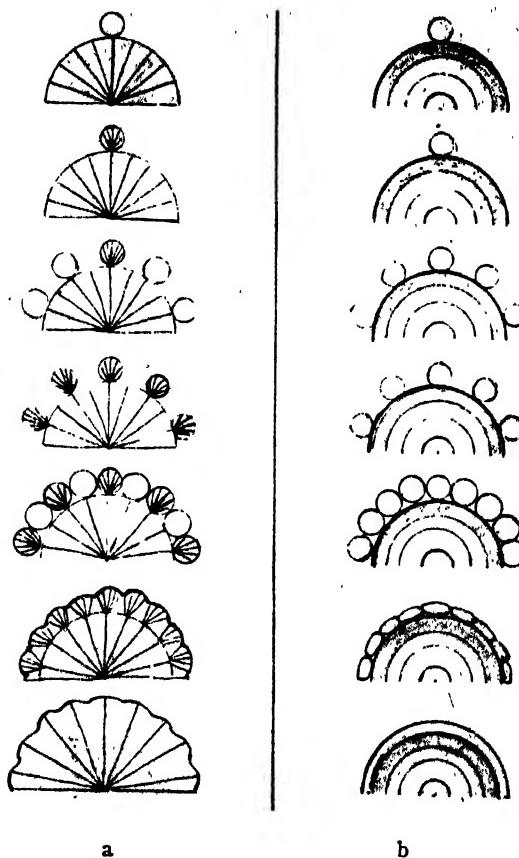
Fig. 37.—Diagram of concrement formation (H. Schade).

a. Course of development of stone growth in guttulate separation of a crystalloid: pure crystalloid stone with simple radial crystal rays.

b. Course of development of stone growth upon separation of a protein colloid: pure protein stone with concentric deposition in layers.

show mutual splitting off of layers by attrition. It is doubtful if finished stones can be forced close together by pressures existing in the human body. The author believes that this is not unthinkable in the case of concretions as hard as ordinary gall-stones at 37°. The effect of long continued slight pressure is usually underestimated: even a seemingly solid stick of sealing-wax supported at one end, bends double in course of time under its own weight.

Liesegang's rings have recently been discussed in connection with concre-

ment structure. If the initial formation of layers in concrements were by apposition, it would then be possible to have Liesegang's rings develop from secondary processes. The author believes that such a view is not tenable. In order to have Liesegang's rings develop in true form, that is, in regular lines, the diffusion must take place in a jelly or gel which has no points of resistance to diffusion. But the colloid framework of the layers contains such points, due to the large amount of crystalloid precipitate irregularly included. Should a process resembling the Liesegang type occur secondarily, very irregular lines must be expected; but they are not found. This argument alone is enough to rule out Liesegang's rings as the cause of concentric structure.

In concluding this section, let us try, by considering the various facts, to group them into a single idea of concrement growth. Initially, the two modes of formation are quite similar: In case of guttulate separation of crystalloids, tiny fluid drops form into layers, as the author has observed microscopically with uric acid and cholesterin. In case of precipitation of albumin, there are fluid to gelatinous droplets of ultramicroscopic size. Both methods of stone-formation begin, therefore, by layering of drops. Figure 37 shows diagrammatically the subsequent development of both methods: to the left, guttulate separation of a crystalloid; to the right, precipitation of albumin. Different properties of the drops cause differences in development. Where a crystalloid droplet touches the stone, it quickly forms sheaf-like crystals. Observations of H. Schade, illustrated in Figure 38, show that this is closely related to the well-known formation of double sheaf crystal. For the reasons given above, the "young" layer of crystals is absorbed sooner or later by the main mass.

FIG. 38.—Types of crystal deposition. (a) In single drops (spherolith). (b) In double drops (double sheaf). (c) By attachment to a spherolith (after H. Schade).

Layers of colloid albumin develop quite differently. There is practically no tendency to crystallization, and the droplets may maintain their gelatinous state for some time. New droplets may come in, complete the ring and cohere. "Aging" sets in; the droplets lose water and shrink, causing a tangential pull on the ring, and leading to a flattening of the droplets and the ring. Repetition of this microscopic procedure leads to the formation of a macroscopic mass of layered structure. We may differentiate, therefore, between crystals and concrements thus: *Concrements are structures whose characteristic form is due basically to the existence of an intermediate guttulate separation.**

III. Concretions of Animal Origin

Concretions are widely distributed in the animal kingdom. As far back as the middle of the last century, Meckel von Hemsbach, in his "Mikrogeologie" before mentioned, had stressed the morphological relation of all layered stones

* In many instances, if not in all, crystal formation begins with the aggregation, into spherulites of molecular groups, which may be tiny crystals or quite amorphous, providing these groups hav sufficiently diminished Brownian motion. The spherulites may then form groups or dendrites, whic later undergo molecular rearrangement into a large crystal. This process is especially marked in sulfu See J. Alexander, First Colloid Symposium Monograph, Madison, Wis., 1923; and Vol. I, this serie

found in animals, and pointed out their relationship to human biliary and urinary stones. The same principles govern the formation of all. They all appear in a fluid-cavity independently of any pre-existing cellular structure; they grow by apposition, and as Figures 39 and 40 indicate, show structurally each of the main types of stone-formation—the concentric layering of true colloid concretions, the radially-striated form of true crystalloid stones, and the combination of the two.

Pearls are a particularly important example of a stone produced in animals by disease; Meckel von Hemsbach called them "the most regular and finest ideal concretion." They arise in certain shell-fish when foreign bodies or parasites get under the shell, causing a morbid separation of the mucous membrane.[35] Chemical analysis by G. and H. S. Harley shows: calcium carbonate, 91.72 per cent; organic matter, 5.94 per cent; water, 2.23 per cent; undetermined, 0.11 per cent. Different kinds of pearls vary in composition. On dissolving out the $CaCO_3$ with dilute acid, there remains a visible organic framework.

FIG. 39.—Type of simple layering—a chitin concretion from the interior of the body of *Branchipus Grubei* (after F. Alverdes).
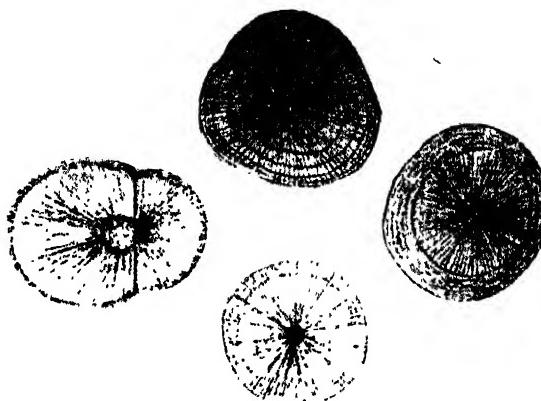
FIG. 40.—Types of radial striation and of the combination of radial striation and stratification—urinary spheres from snail kidney (*Helix pomatia*) (after E. Korschelt). Enlarged 600x.

Figures 41 and 42 show two different types of pearl structure, apart from the fact that one has a double kernel. Referring to Figure 26, we would say that pearls having radially disposed outer layers are dull and lusterless, while pearls having the structure shown in Figure 42 show the characteristic "pearly luster."

Actual practice was centuries ahead in this branch of science. As far back as 1656, Jaquin, by making thin layers with colloids and crystalloids, made

[35] See article on "Pearls," by E. Korschelt, in E. Abderhalden's "Fortschritte der naturwissenschaftlichen Forschung," 7, 111-190 (1913). No reference is made here to physical chemistry.

artificial pearls that deceived all buyers. The "Oriental Essence" he used is
a suspension of tiny crystals of calcium guanin, obtained by triturating the
silvery scales of white fish (*Cyprinus alburnus*). A mixture of this suspen-
sion with gelatin is introduced into the interior of glass beads to form, when

FIG. 41.—Section of a pearl of *Margari-*
tana margaritifera (after E. Kor-
schelt). Enlarged 4x.
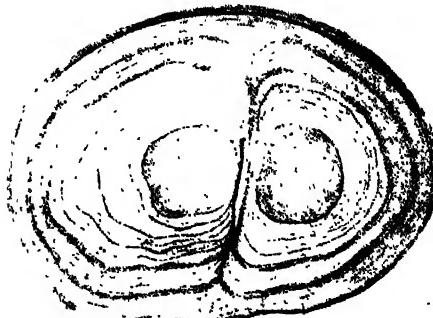
FIG. 42.--Section of an American fresh-
water pearl consisting mainly of
"mother-of-pearl" after E. Korschelt).
Enlarged 5x.

dry, a thin layer. After several superimposed layers are thus formed, the
interior is filled with wax, giving a sphere remarkably like real pearl. Even
to-day, this old tried-out method is in use, the gelatin being mostly replaced
by nitrocellulose or the like (O. Wilhelmy).

FIG. 43.—Statocyst of *Pterotrachea Fried-*
erici, coated by plates of hair-carrying
ciliated cells, and above by cells with
sensory hairs. The statolith is in the
center.
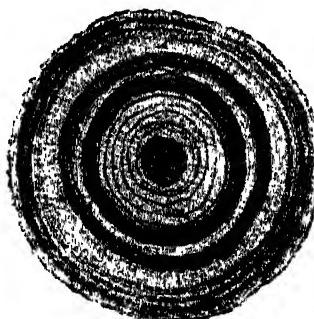
FIG 44.—Gastrolith (so-called crab-stone)
from the stomach-pocket of a decapod
(after E. Korschelt). Section, enlarged
7½x.

Though the concretions heretofore considered are morbid structures, other
kinds may be physiologically useful; e.g., *otoliths* (ear stones) and *statoliths*
(central structures in balancing organs). These occur in various but typical
concretional forms in medusae, worms, crabs, molluscs, and also vertebrates
(see Fig. 43). Similar in form are *gastroliths*, formed in crabs in a side

pocket of the stomach, by a co-precipitation of crystalloids (calcium carbonate and phosphate) with colloids (Fig. 44). These "crab-stones" are formed by the organism in the course of years to furnish an available supply of lime at molting time, for sea water has but little lime. When the new shell is to form during molting, the "pocket" opens inward to the stomach, and the stone is dissolved by the hydrochloric acid of the gastric juice. At each shedding, this process is repeated. This is astonishing to the physician, for it shows what he has long striven to accomplish—the re-solution of a stone within the organism itself.

As Meckel von Hemsbach first pointed out, it is probable that there is a close connection between concrement formation and many animal shells, e.g., those of shell-fish, which, though free of cells, are built up from a special cellular secretion. Here, too, crystalloids and colloids are combined. Figure 45 shows that these shells show the three main types of concrement structure. The deviation from spherical form may well represent accidental factors: growth of a shell takes place by apposition on the hollow side, where there is formed an under layer with continually increasing scales. Before considering these structures as concretions, we must first settle the question as to whether they are not incrustations of precipitated colloids, with crystalloids deposited in them. Quite similar in form and origin are fish scales, turtle shells, and like structures found in diverse forms in various animals. Sometimes these resemble Liesegang's rings. Each case must be decided on the basis of its own evidence.

Sometimes the *human skin* shows in its cell-free horny layer structural changes which suggest those found in the formation of concretions. Figure 46a shows the normal skin; the basal horny layer, containing the most recently formed horny substance, appears microscopically as a homogeneous clear layer

Cardinia hybrida

Orthis Dumontiana
(interior view)
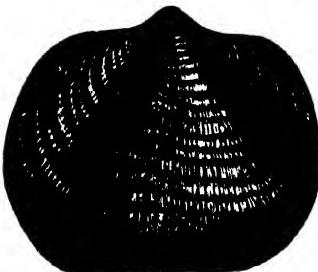
Productus semireticulatus

Fig. 45. — Typical concrement structures in shell-fish (after J. Leunis).

(B.H.). Parallel layer formation appears later in the dried super-imposed horny layer (H). The behavior of the horny substance in Figure 46b is especially significant, for it shows the operation of the general laws of colloid formation in concrements (excluding the region dominated by the living epithelium, E)· aggregation to concentrically layered spheres, resembling so-called "horn pearls." Morbid retention of horny masses in the human skin often leads to the formation of such "horn pearls."

FIG. 46a. FIG. 46b.

FIG. 46.—a. Normal human skin, with usual layers of horny material. b. Skin in a human cyst, showing formation of "horn pearls" (after H. Ribbert). H = horny layer; B.H. = basal horny layer; E = epithelial layer; B = connective tissue layer.

IV. CONCRETIONS OF VEGETABLE ORIGIN

Regarding concrements in the vegetable kingdom but little is yet known. According to the literature compiled by E. Korschelt[39] there are in the cocoanut, the bamboo, and the fern, bodies which closely resemble in appearance and structure animal pearls. G. Kohl[40] has also described spheroliths of
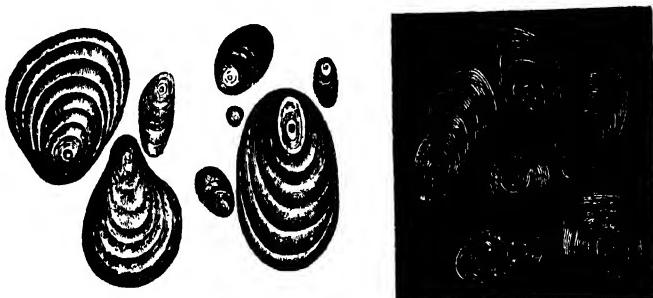
FIG. 47.—Starch grains of potato. (Enlarged 350x; right, in polarized light.)

microscopic size, with concentric layering and radial striation, which he has found in various parts of plants. Chief reference is to be made here to starch grains as the most important plant structure of concrement nature.

Figure 47 shows starch granules of the potato with their typical concentric layers.

Figure 48 is added here because it affords proof that the layering of these starch granules depends, not upon a Liesegang's ring structure, as has been

[39] Korschelt, E., "Pearls" (old and new things about their structure, or origin, and use) in Abderhalden, E., Fortschritte naturwiss. Forsch., 7, 111-190 (1913).
[40] Kohl, G., "Anatomisch-physiologische Untersuchungen der Kalksalze und Kieselsäure in der Pflanze," Marburg, 1889.

hitherto supposed,[41] but upon appositional growth. The dark-granuled chlorophyl-containing plasts are distinctly visible as they build up the separate starch granules at the poles of the largest masses. These chloroplasts add layer on layer of new starch-colloid.[42] The presence of the polarization cross (Fig. 47) is an evidence of the stress occurring in the several layers in consequence of the shrinking of the colloid with age (compare Fig. 37 and accompanying
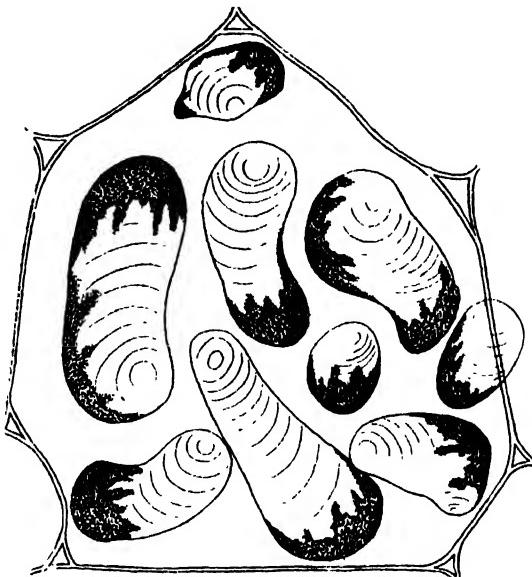
Fig 48—Starch grains with green chlorophyl granules, from the stem of *Pellionia davcauana*. After R. Kolkwitz. (Enlarged about 350x.)

text) ; if the starch grains are allowed to swell in water, this optical appearance is lost (A. Meyer).[43]

V. Concretions of Mineral Origin

Large masses of earthy stones have been formed by precipitation from aqueous solution. The petrography of sediments, which undertakes to describe these masses, gives us an idea of the various chemical substances which participate in their formation. These are in part crystalloids, and in part materials like silicic acid, ferric hydroxide, and others, which could hardly have precipitated from aqueous solution otherwise than as colloids.

The physico-chemical explanation of the laws of concrement formation, as hereinbefore described in detail, is certainly of equal importance as applied to these mineral precipitations.

Geology, that branch of science which should deal with these matters, is

[41] See, for example, Bechhold, H , "Die Kolloide in Biologie und Medicin," Dresden Leipzig, 2nd ed., seite 285.
[42] Dodel, A., in *Flora*, **75**, 267-280 (1892)
[43] Meyer, A., "Untersuchungen uber die Starkekorner," Jena, 1895

to-day almost a stranger to investigations of this kind. The explanation of the structure of the Carlsbad pea-stones and of the Lorraine roe-stones or oölites, by tracing their origin from a combined colloid-crystalloid precipitation, as shown experimentally by the author [44] in 1909, and shortly thereafter accepted by specialists,[45] is, indeed, mentioned as an established isolated fact in geology,[46] but there has been no disposition to utilize more extensively this theory of concrement structure for the understanding of sedimentary stones. An essay in this direction will be given in the following explanation.

Figures 49 and 50 represent these two mineral concretions, whose origin

FIG. 49.--"Oöids" of Lorraine roe-stones. Photomicrograph after H. Schade. (Enlarged 100x.)

and structure are made clear in the above-mentioned work of H. Schade. In both stones, after dissolving out the crystalloid portions (calcium carbonate, particularly), there remains a distinct colloid skeleton (silicic acid or ferric hydroxide). If due allowance is made for the chemical difference of material, we find here complete analogy of structure with, for example, urinary concretions.

Similarity of structure connotes similarity in conditions of formation. The formation of the Carlsbad oöids is still going on and may be observed closely in detail. Colloids (silicic acid and ferric hydroxide) together with crystalloids (especially calcium carbonate) supply the material. By reason

[44] Schade, H., loc. cit.
[45] Kolloid-Z., 4, 277 (1909) (E. Dittler); idem., 4, 290 (F. Cornu and H. Leitmeier).
[46] Compare, for example, C. Doelter, "Handbuch der Mineralogie."

of the escape of carbon dioxide from the water as it flows from the spring, there occurs almost immediately supersaturation for the above-mentioned substances, and, later, precipitation. If it happens that, during this precipitation, there are present, in the basin of the spring, sand grains, for instance, held in suspension in the whirling waters, or even larger pieces of stone (see kernel of oöid in Fig. 50) that may serve as adsorption centers, then are satisfied the conditions for the formation of concretions on these bodies as nuclei; and thus, from the mixed precipitate of colloids and crystalloids, concretions are formed with concentric layering, and crystalline radiation due to criss-crossing of layers. All foreign bodies with adsorbing surfaces, which are held in suspension in the sprudel-water, also flowers or other parts of plants, may become centers of concrement structures. In such concrement formation, the period during which the

FIG. 50.—"Oöid" of the Carlsbad sprudel-stone (=pea-stone) after H. Schade. (¾ natural size.)

concretion may grow, on all of its faces, is limited. When the concretion can no longer be held in suspension, it sinks to the bottom and there finds a suitable place to lie—thereafter growing no longer on all sides.

FIG. 51.—Oolith: typical pea-stone of Carlsbad, after H. Schade. (¾ natural size.)

Extensive deposits of such concretions are frequently found; composed of oöids, they are usually called "ooliths." As the size to which the oöids can grow, while held in suspension, is limited by the local conditions of the water (the velocity of its whirling motion, and so forth), it is readily understood why the oöids collected in a given place and united into an oölith are of nearly the same size. (Compare Fig. 51.) Ooliths in different localities generally show, on the other hand, different sizes of oöids; their size varies from large structures (see, for example, Fig. 50, above), down to pea-sized oöids (see

the typical pea-stone of Carlsbad in Fig. 51), and even to the size of fish-eggs (see the roe-stones of Fig. 49), and finally to those of microscopical size (in the so-called "Cryptoliths"). Oöliths are widely distributed in nature. In their composition are found, in addition to silicic acid and iron hydroxide (and other oxides produced therefrom), manganese oxide (or dioxide), gypsum, and other materials; colloids are always present, and according to E. Dittler and F. Cornu,[47] are demonstrable in stained microscopical preparations. Not only in Carlsbad, Vichy, and other health-resorts is recent oöid formation known, but also in the Suez Canal, the Wady of Deheese at Sinai, and along the coast of Florida. In prehistoric times oöid and oölith formation was very frequent; in many strata of sedimentary rocks, especially of the Jurassic period, during which some strata were predominantly formed in this manner, and also in the still more ancient Triassic and Silurian times. The Lorraine roe-stone (Fig. 49) is an example of this. Investigations concerning the occurrence of oöliths in the sedimentary rocks of the earth's crust have been published by W. von Gümbel [48] and, more recently, by W. Wetzel.[49] G. Linck,[50] E. Kalkowski,[51] and lately A. Rothpletz and K. Giesenhagen [52] have produced works which deal with the origin of oöliths. Both Kalkowski and the last named authors are of the opinion that bacteria have played a definite rôle in the formation of oöids. The investigations of Schade, referred to above, show, however, that the origin of oöids and oöliths is, *eo ipso,* possible without any bacteria. Furthermore, the non-bacterial origin of the Carlsbad pea-stone is certain; for the author, through the kindness of Dr. R. Kampe, has been able, in the closed and unquestionably bacteria-free (at 73° C.) vent-pipe at Carlsbad, to observe, under most favorable conditions, a typical oöid, during its growth.

These mineralogical oöids furnish a good example of the enrichment of the concrement by adsorption, in consequence of which the relative percentages of the materials in the concretion may differ widely from what we find in the solution. Thus the Carlsbad pea-stone, which comes from a water containing 0.032 per cent calcium carbonate and 0.0071 per cent silicic acid, may contain as much as 52 parts of silicic acid to 100 parts of calcium carbonate; and in a spring at Baden it was observed that, from a water containing 0.01151 per cent of calcium carbonate and 0.0119 per cent of silicic acid, a deposit was formed which showed the high figure of 449 parts of silicic acid to 100 parts of calcium carbonate. But this is a peculiarity which mineralogical concretions have in common with renal and biliary stones (H. Schade).

As we saw above, in speaking of human concretions (compare Fig. 27), in addition to the formation of concrements free in the various cavities, it is possible to have *formation of precipitate on the wall of the mucous membrane and attached thereto.* There exist, therefore, forms of concretions which may be called *"tube-casts."* In those cavities of the animal body which are lined with mucous membrane, such process takes place only exceptionally—that is, only when the mucous membrane, through pathological degeneration from its normal condition, shows a marked tendency towards adsorption. But this, which is an exceptional occurrence in the mucous cavities of the living body, becomes in inorganic nature the rule, for here there are scarcely any limiting

[47] *Kolloid-Z.,* **4,** 277 (1909).
[48] v. Gümbel, W., "Grundzüge der Geologie," Kassel, **1888.**
[49] Wetzel, W., "Sedimentpetrographie," *Fortschr. Mineral.,* **8** (1923).
[50] *Neues Jahrb. Mineral., Palcontol.,* **16,** 495 (1903); *Jenaische Z. Naturwiss.,* **45** (1909).
[51] *Z. deut. geol. Ges.,* **60,** 68-125 (1908).
[52] *Abh. Bayer. Acad. Wiss., Mathem. physik. Kl.,* **29,** 5 Abt., p. 1-41 (1922).

walls, that, in a practical sense, lack adsorptive ability. The usual concretions on the walls of the large iron pipes through which the Carlsbad Sprudel water is conducted, may here serve as an example (Fig. 52). On traveling long distances in the pipe, diminution of pressure occurs, as is usual in spring
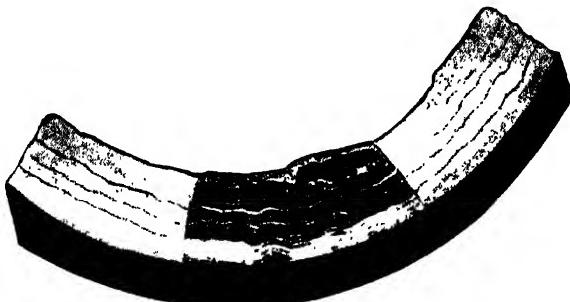
FIG. 52. "Tube-casts" from Carlsbad Sprudel. (Formed in a few years.) After H. Schade. (About ⅓ natural size.)

water brought from great depths, by reason of violent effervescence of carbon dioxide, so that far on in the pipe the conditions are present for simultaneous precipitation of colloids (silicic acid and iron hydroxide) and crystalloids (especially calcium carbonate), and, therefore, concrement formation takes place in a way decidedly disturbing to the operation. The structure which is typical under such conditions, concentric layering and radial striation, is here found particularly well exemplified. Such tube-casts differ from the "oöid" (Fig. 50) only by reason of the gross mechanical differences in the conditions under which the layers are formed. The same conditions, which to-day produce these concretions on the walls of the exit pipe of the spring, have in former ages (in the Tertiary presumably) operated to form similar deposits in a large way, i.e., on a geological scale, around the walls of an immense confined lake at Carlsbad. The "sprudel-shell" produced at that time is still recognizable to this day, on the mountain cliffs of the Carlsbad valley, extending even up to the plateau in massive remnants, and the whole inner city of Carlsbad is built upon the concrement which was once deposited on the floor of this lake to the depth of 10 meters or more (T. Noeggerath,[53] R. Kampe [54]). Such crustaceous concrements on a large scale are usually called in geology "stromatoliths." In Figure 53 (photographic reproduction of the plane surface of a small portion) is shown a picture of the structure of this

FIG. 53.—Stromatolith from the prehistoric dammed-up lake of Carlsbad Sprudel. After H. Schade. (¾ natural size.)

[53] Noeggerath, T., "Die Sprudelschale in Karlsbad, 37 Versammlung deutscher Naturforschen und Aerzte." Karlsbad, 1862.
[54] "Internationale Fortbildungskursus in Karlsbad," 3, 258 (1922).

gigantic concrement-shell of the old Carlsbad basin, which is very closely related genetically to the present-day Sprudel-stone, i.e., oöid.

Similar geological formations, with structure typical of origin from a concrement process, are undoubtedly to be found in many other places. Conclusive proof of origin through one of the processes of concrement formation here mentioned, can always be found if, in such stromatoliths, the existence of oöids, of related material and structure, can be demonstrated. For not a few geologically important sedimentary strata this proof may already be ad-

FIG. 54.—Materially- and structurally-related Stromatolith- and Oöid-formation in one and the same stone (North German colored sandstone). After E. Kalkowski.

duced from observations of their materials as described in the literature; for example, especially in the North German colored sandstone, as the reproduction (Fig. 54) of a beautiful illustration of E. Kalkowski (*loc. cit.*) shows.

Some of the silicic acid and calcium carbonate deposits, including special forms found in subterranean caves as "stalagmites" and "stalactites," are to be regarded as concrements. They show typical stratification of colloidal origin, and often a crystalline radial structure (cf. W. v. Gümbel, "Grundzüge der Geologie," Kassel, 1888, Fig. 173, page 247). Especially significant does it appear to the author, that in the center of the stalagmite at its upper end, in the bowl-shaped cavity where the liquid from the ceiling drops and stirs the solution from time to time, can occasionally be found oölithic lime concrements (cf. A. Tornquist, "Geologie I," page 167. Leipzig, 1916).

The formation of these geological concretions (abstracting from the formations of to-day, the so-called recent formations) has taken a great deal of time. This fact is to be carefully noted. For as we saw above in human gall-stones, for instance, concrements can, under the influence of age, undergo very decided changes not only in their crystalloid but also in their colloid constitution. When we consider the speed with which changes due to age
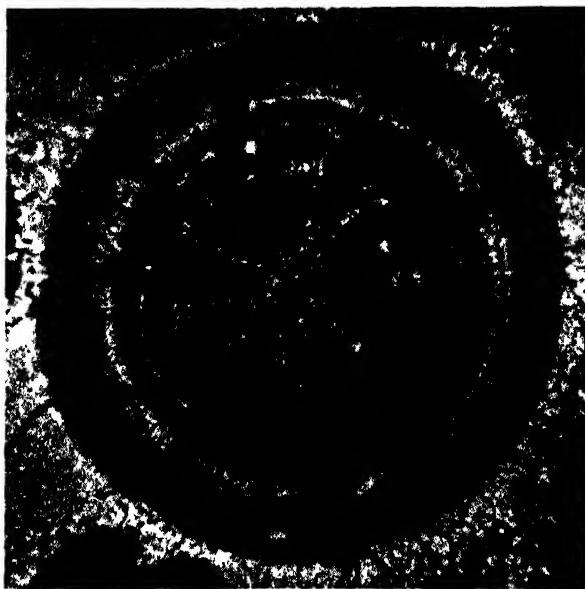
FIG. 55a.
Oöid with fully preserved layer formation in rings.

FIG. 55b.
Oöid (by accident double) with secondary crystalline rupture of ring layers as a result of age.

occur in cholesterin stones we realize that in Geology it is even more neces-
sary to take into account very great secondary alterations in these concre-
tions.   Possibly such formations exist in the sedimentary layers of the earth,
but it would be very difficult, at least in the present state of our knowledge, to
recognize them as such.   The speed with which these formations were built
up during past ages was, as was shown by the comparison of cholesterin stones
with other human concrements, subject to extreme variation.   It is important
to study the phenomena of aging in geological concrements as well.   It is
very interesting that geological concrements show exactly the same types of
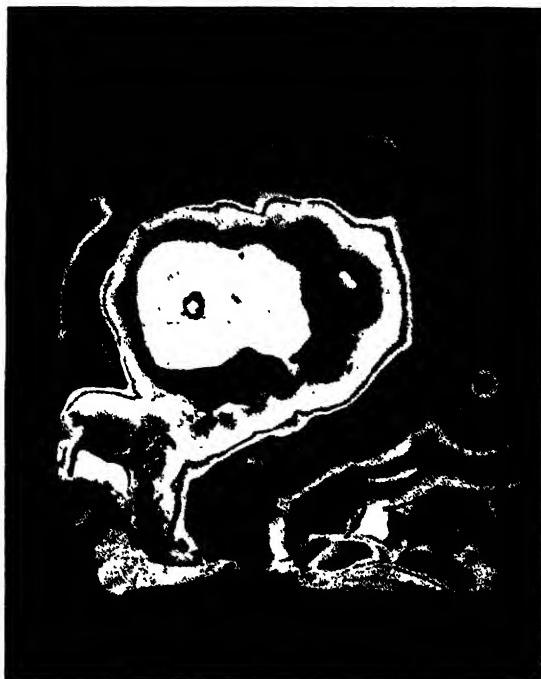
FIG. 56.—Carlsbad sprudel-stone sectioned tangentially to the layers.   After R. Kampe.

secondary formations as we found in bile-stones due to typical aging-processes.
This is shown in figures from geological literature.   Figure 55, after E. Kal-
kowski (*loc. cit.*) shows, for mineralogical oöids, in exactly the same way as
the cholesterin stones of Figures 35 and 36, the secondary crystalline forma-
tion with resulting rupture of the original lines of stratification, by radial
striations.   The effects of this secondary change of colloids are plainly visible
in geological concretions, and the appearances are of the same type as we
find in human concretions due to age: radial rending of the concrement mass,
beginning in the interior and often remaining localized in the innermost strati-
fications, but frequently penetrating through the entire concrement, so that
remnants, broken away therefrom, may be found lying in the secondary stone
mass.   This happens so often (cf. E. Kalkowski) that an illustrative figure
is unnecessary.
    To judge these geological formations it is necessary to cut through the

stone in the proper way. Figure 56 shows a Carlsbad sprudel-stone that is cut, not vertically as in Figure 53, but horizontally. It is remarkable how similar such a photograph is to that of an agate, i.e., a stone whose stratification originated by diffusion after the type of "Liesegang's rings."[55] The characteristics of the formation of concrements by apposition as distinguished from secondary Liesegang's stratification through diffusion, will now be given:

1. In concrements the adjoining layers are composed of materials which differ from one another structurally and chemically; in the case of Liesegang's rings, the basic substance is uniform and by impregnation of this mass with the foreign material, rings are formed at definite distances apart, thus dividing the mass into sections.

2. If the growth is appositional the distances between different layers are irregular. In Liesegang's rings there is always a remarkable rhythm in the distances of the layers.

3. In the case of appositional growth the colloids or crystalloids of the
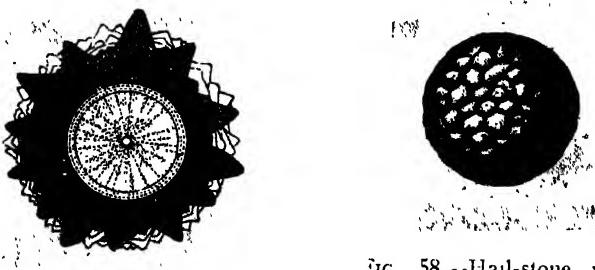
Fig. 57. — Hail-stone of spherolithic structure.

FIG. 58.--Hail-stone with typical studding of the surface as evidence of its origin by guttulate separation.

outer layers, especially the outermost, often show signs of "youth." In the case of Liesegang's rings we often find a gradual disappearance of the strength of the phenomenon, instead of such a qualitative distinction in structure.

4. The boundaries of the stratification are for the most part very sharp in the case of appositional growth. The borders of Liesegang's rings are often not equally well marked on their inner and outer margins and in that case all the borders lack sharpness on the same side.

5. In appositional growth there can be large particles in one layer without notable effect on the structure of neighboring layers. In Liesegang's rings interference with diffusion causes disturbance of many rings. If in a concrement formed by colloid-crystalloid apposition, Liesegang's rings later occur, there is, because of the many points that act on diffusion, a decided disturbance of the Liesegang lines and of necessity frequent intersection with the lines of the layers. In concretions such conditions are not found and therefore additive Liesegang's rings are absent.

The above-mentioned mineralogical concrements are formed from combined colloid and crystalloid deposits. *Pure colloid stones* with banded structure are very rare. But if the term "purity," as in our consideration of concretions of the human body, be taken not too literally, then we may include here many deposits of silicic acid and silicates with, at times, massive stratifica-

[55] Cf. Liesegang, R. E., "Die Achate," Dresden-Leipzig, 1911.

tion.   But the third group of concrement formations, namely, the *pure crystal-loid stones,* originating in guttulate separation, has its representative in min-eralogical specimens.   As H. Schade has demonstrated,[56] hail-stones are a very good example of this group.   There can be no doubt that, in hail-stones, we have guttulate separation with air as the medium.   As the reproductions of the well-known illustrations 57 to 59 show, the structure of hail-stones is very similar to that of the cholesterin solitary stones and of uric acid stones, both

Fig. 59.—Hail-stones.   Photomicrograph.   (Enlarged about 12x.)

in radial striation and in the humping of the surface which is characteristic of formation from guttulate separation.   ·

Concerning the cause of the stratification clearly to be seen in Figures 57 and 59 evidence is still lacking.   It is probable that stratification is here pro-duced by temporary interruption of the process of formation; perhaps there is, in addition, an intermediary colloidal form of water,[57] which has its shaping influence.

The physico-chemical doctrine of concrement formation is, at the present time, highly developed, but many questions await further investigation, and the full possibilities of its practical application are by no means fully realized.

56 *Kolloidchem. Beih.,* 1, 387 (1909).
57 Schade, H., *Kolloid-Z.,* 7, 26 (1910).

Dusts, Fumes and Smoke and Their Relation to Health

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The earth's atmosphere consists of a mixture of nitrogen; oxygen; carbon dioxide; certain rare gases, such as helium, neon, krypton and argon; water vapor and a certain amount of other liquid matter, chiefly in the form of tar; and lastly, solid matter which is usually referred to as dust. This solid matter has as its origin the soil constituting the earth's surface; particles of plant or animal substance, including pollens and grains; salts, whose origin as a rule is spray from the ocean; volcanic dust; products of combustion; and products of abrasion.

There exists a small fraction of air-borne dust which may remain more or less permanently in suspension in the air. The distance to which this may be carried is limited only by the limits of the atmosphere. The heavier fractions of dust are carried relatively shorter distances, but evidence which appears trustworthy indicates that even this heavy material may be carried a distance of 1,000 or 2,000 miles.[1] Dust storms originating in the Sahara Desert and traveling over Southern and Central Europe have been observed in Northern Germany and in England, a distance of some 2,000 miles.

When one considers the enormous range which exists in the composition, size and concentration of dust, one must be convinced that the animal and the human lung must be the repository for a vast amount of material of varied kinds. The air filtering equipment of the body is but a partial protection against such inhalation.

The total amount of dust present in the air at any one time varies over a wide range. In fact, at any given instant the amount of dust present will be found to depend on the particular instrument used for the sampling and estimation of the dust. And so many instruments have been used for this purpose that in evaluating dust counts one must exercise care to bear in mind the particular instrument used for making the estimation.

In 1875, Coulier [2] showed that the moisture in air could be forced to condense on dust particles when the pressure in the containing vessel was suddenly reduced. This principle were utilized by Aitken a few years later in the instrument called the Koniscope and in the dust counter. Utilizing this method, Aitken found that the number of particles per cubic inch of country air varied from 8,000 to 100,000, whereas in towns it varied from 1,000,000 to 50,000,000. The counts shown on the following page were obtained by Doctor Cohen [3] with this instrument and confirm the findings of Aitken.

It will be noted that according to the above table the air of the Leeds flour mill contains less dust than the outdoor air of the Paris churchyard. This is without doubt due to coalescence and aggregation of many of the dust particles in the case of the flour-mill dust. Apparently it was thought that an instrument such as the dust counter sampled all the dust particles present, the size

[1] Free, E. E., "The Movement of Soil Material by the Wind," *U. S. Dept. Agric. Bull.*, **68**, Washington (1911).

[2] Coulier, *J. Pharm.*, **22**, 165 (1875).

[3] Cohen, J. B., "The Air of Towns," *Smithsonian Miscellaneous Collections*, No. 1073 (1896).

Dust Particles in Air	Number of Particles
Place where air sample was taken—	per Cubic Inch
Woodhouse Moor, northwest wind......................	530,000
Tennis Court, Yorkshire College.......................	852,000
Town Hall Square, Leeds..............................	1,228,000
Paris churchyard, Leeds	3,638,000
Glasgow Town, northwest wind (Aitken)................	3,736,000
Flour mill, Leeds....................................	3,113,000

of each particle being increased by the droplet of moisture condensing thereon and so becoming visible to the observer. We need not concern ourselves about the exact degree of truth of these ideas; for such a complete discussion reference may be made to Barus.[4] It is safe to say that this instrument reveals the general order of magnitude of the number of dust particles present in the atmosphere.

Taking into account the whole gamut of dust sizes, we may profitably consider the revision by Drinker and Thomson[5] of Gibbs'[6] classification of dusts, fumes and smokes.

"Dusts.—Particles or aggregates of particles, 150 to 1 micron in diameter, that are thrown into the air by mechanical agencies are termed dusts. Examples are silica, talc, cement, organic dusts such as hard rubber, starch, and cocoa; flocculated fume and smoke products.

Fumes.—Particles, 0.2 to 1 micron in diameter, formed from chemical or physicochemical reactions are known as fumes. Examples are ammonium chloride, lead and mercury, zinc and magnesium oxides, fogs, and acid mists.

Smokes.—Particles, less than 0.3 micron in diameter, formed by incomplete combustion of carbonaceous and other substances are called smokes. Examples are the particles formed from burning tobacco, oil, tar, and gas."

It will be observed that, according to this classification, the particle sizes range from approximately 150 microns down to a size less than 0.3 microns. The effects of these various sized particles on health may be considered under these three headings, for those particles of the first group between 0.5 and 15 microns may be associated with certain respiratory diseases and conditions of hypersusceptibility. Those between 0.2 and 1 micron in diameter may be associated with certain systemic poisons. And lastly, those particles approximating 0.3 microns in diameter and less, which are formed by the incomplete combustion of fuels, are possibly associated to an unknown degree with alterations in the respiratory disease rate and most largely associated with the absorption of certain rays of the sun, beneficial to health.

When one takes into consideration dust particles of all sizes, including those particles which are so small as to be rendered visible only under the highest powers of the microscope, or by such means as the Aitken Koniscope or dust counter, it is obvious that man must, even in a very brief time, inhale particles in enormous numbers. Just how much dust may reach the lung tissue is not known, but that there is an upper limit to the size of particles which may enter the lung is without doubt true. The South African investigations have been highly informative on this question. In 1913 Dr. J. McCrae[7]

[4] Barus, in Alexander's "Colloid Chemistry," Vol. I of this series, p. 420, New York (1926).
[5] Drinker and Thomson, *Trans. Am. Inst. Mining Met. Eng.*, March (1925).
[6] Gibbs, *J. Soc. Chem. Ind.*, 41, 189 (1922); also paper in Vol. I of this series.
[7] McCrae, *South African Inst. Med. Research*, Publication I, Johannesburg (1913).

separated the siliceous particles from a silicotic lung and subjected these to a microscopic examination. The majority of the particles were found to have a diameter less than 1 micron, in fact 70 per cent of the material was in this size group. Of the remaining material the majority was found to have a size between 1 and 8.5 microns, and the very largest particle observed was 10.5 microns. In addition to these data, Dr. Watkins Pitchford has measured the dimensions of the particles of dust in sections of silicotic lung mounted in Canada balsam.[8] The length of all of the particles with one exception fell between 1.1 and 13.0 microns (the exception was 24 $\times$ 30 microns in size) and but seven of the 101 particles were over 10 microns in size. Concerning large particles, Dr. Watkins Pitchford says:

"As necrotic areas in silicotic lungs frequently include the disintegrated remains of air-vesicles and bronchioles which have become devitalized by obliteration of blood-supply, the presence in them of large particles does not necessarily indicate that such particles had actually penetrated the limiting walls of the alveoli and air channels. Inspection of specimens in the earlier stages of the condition shows indeed that the double-refracting particles which quit the air spaces to enter the connective-tissue, are all of small size and nearly all of apparently elongated shape. It is probable therefore that the large, plate-like particle which comes third in the table had never actually entered the lung tissue."

And lastly, we may refer to the examination by Dr. J. Moir of a specimen of dust from silicotic lung.[9] He obtained two specimens of lung dust, one by incineration with $AmNO_3$ followed by weak HCl, and a second by a digestion with HNO_3. One hundred and twenty successive particles without selection were examined and measured. All were found to be less than five and one half microns in longest dimension and seventy-three were one micron and smaller in size. It is largely on the basis of these studies that workers in this field have concluded that particles more than ten microns in size play but a minor rôle in the causation of silicosis among miners in South Africa, and it is very likely that for all practical purposes particles of such size play an unimportant part in the causation of pulmonary disease, no matter what the composition of the dust. There is little reason to expect that a particle of other material should meet a different fate at the hands of the filtering mechanisms of the nasopharynx than should the dust of the South African mines. If this is so, we may for all practical purposes consider dust of a size over 10 microns as relatively unimportant in our discussion of this subject.

Concerning the lower size limit of the dust particles which may have hygienic and sanitary significance there is less known. As noted earlier, the number of dust particles of a small size, from say 1 micron to a size just visible under the high power microscope, which are present in the atmosphere must be very large. That large numbers of such particles are inhaled with every breath must be the fact. Assuming this to be the case, we must next concern ourselves with the problem of deciding as to how much of this material really is taken up by the lung tissue and remains there to bring about pathology in this situation.

Some interesting studies have been made on the retention of dust by the

[8] Watkins-Pitchford, "General Report of the Miners' Phthisis Prevention Committee," Pretoria, 1916, Appendix 8, p. 135.
[9] Moir, "General Report of the Miners' Phthisis Prevention Committee," Pretoria, 1916, Appendix 9, p. 138.

.lungs. Those by Lehmann and his associates indicate that from 40 to 79 per cent of white lead dust is caught in the lungs, while the studies of Bamberger indicate that from 67 to 88 per cent of tobacco smoke is caught by the lungs, depending on whether the subject is puffing or inhaling the smoke. The excellent studies of Drinker and his associates disclosed the fact that approximately 55 per cent of the inhaled material is retained by the lungs (two of the dusts used were 0.4 and 0.16 microns in particle size). Lastly, mention must be made of the studies of Sayers, Fieldner, and their associates, who in investigating ethyl gasoline found a 15 per cent retention in the respiration of very low concentration lead compounds given off in the diluted gases from an internal combustion engine burning ethyl gasoline. From these data one must be convinced that a large amount of dust is continually being taken into the lungs and of this a portion, perhaps half or more, is retained therein at least temporarily. Of the retained portion a certain amount, just how much is doubtful, may be removed from the lung by the physiological mechanisms, such as phagocytic action and lymph drainage. The remainder probably produces those conditions in the chest of the city dweller which become evident at autopsy, such as darkening of the pulmonary tissue and often a moderate degree of fibrosis.*

METHODS FOR THE SAMPLING OF AERIAL DUST

Before proceeding further with our discussion, it may be well to review briefly the methods which have been used for the sampling of dust in air. It is impossible from lack of space to discuss this subject in great detail. For such a discussion reference may be made to a paper recently published by the writer.[10]

The sampling of atmospheric dust has been attempted by methods based on various physical principles and the methods may well be analyzed on this basis. The chief methods which have been used are the following: condensation, filtration, washing, sedimentation, impinging, electrostatic deposition, and resistance.

The condensation method of which the Aitken Koniscope or dust counter may be considered good examples depends on the condensation of the moisture on the particles by the reduction of pressure in the containing vessel. A recent adaptation of the principle of condensation is utilized in the device of Owens.[11] This device is of great value in the study of dust where the concentration is relatively low, such as in outdoor air.

The filtration principle has been used for the sampling of dust in many forms. Coulier, Cohen, Harcourt, Duckering, and others have used cotton wool for this purpose while Hill and Carrier used cloth filter bags. Disks of canton flannel have been used by Todd, while Gooch crucibles have been used by other investigators. Filter paper has been widely used, both in the form of sheets and in the form of extraction thimbles. Granular solids have been used with a fair degree of success, for example granulated sugar has for a long time been employed by the U. S. Bureau of Mines. Of the filtration methods, those utilizing filter paper are without doubt of much value since the yield is very nearly 100 per cent.

* From the paper of Abramson in this volume, it seems evident that colloidal particles might be drawn into the body by electrophoresis If they entered the circulation, they might give rise to some of the effects described by A. Lumière in his paper in this volume. Experiments along this line would be of interest. *J. A.*
[10] Greenburg, *Public Health Reports*, **40**, 765 (1925).
[11] Owens, *J. Ind. Hyg.*, **4**, 522 (1923).

The washing method, perhaps one of the earliest methods used, is rather low in efficiency because it is after all rather difficult to wet dust with water. Many modifications of this method have been proposed, for example the use of Leibig bulbs by Tissandier in 1874, the Palmer method by G. T. Palmer in 1916, and the Read water spray apparatus at the Broken Hill South Limited in 1924.

Sedimentation of dust on plates, oiled or greased surfaces, has been used in many forms, by Miguel, Tissandier, Des Vouez and Owens and others. Such a method yields a roughly approximate figure for the amount of material which may fall on the surface and adhere thereto in a given length of time.

The impingment method is to some degree a modification of a gravity settlement method, but here the use of high velocity jets may make the efficiency so relatively high that a good dust yield may be obtained. In the konimeter first designed by Kotze of the Miners' Phthisis Prevention Committee, the dust is sampled by impinging it at high velocity, 200 to 250 feet per second, on a vaseline-coated glass plate. This instrument is small in size, portable, and in its newest form is capable of taking 29 dust samples on one slide. The dust catching efficiency of the konimeter is relatively high. The impingement principle is used in a dust instrument designed by the writer and G. W. Smith. This instrument is so designed as to impinge the dusty air on the surface of a wetted glass plate at a velocity of approximately 200 feet per second. The dust is momentarily stopped and wetted and caught in the water which may be removed for analysis. The efficiency of this device is high, for example it has an efficiency of 96 per cent when tested against silica dust gravimetrically, and of 93 per cent when tested optically. When tested optically against tobacco smoke the efficiency is 66 per cent.

The resistance method has only been utilized in one dust sampling device so far as the writer is aware, and in this device the difference in pressure between the two sides of a piece of filter paper is disclosed by means of a U-tube filled with a suitable liquid. The change in pressure with time of flow is noted and rate of change is considered to bear a relation to the dust content of the air.

In the electrostatic method, use is made of the fact that dust particles suspended in an electric field migrate to one of the electric poles. The most improved device based on this principle is that of Drinker, Thomson and Fitchet. It is an exceedingly simple device weighing but 13 pounds, operated by four alkali cells and has a very high operating efficiency, catching practically 100 per cent of the dust passed into it.

For use in those industrial health studies where one is interested in determining the relationship between atmospheric dust and the health of the worker, it becomes necessary to use an instrument which possesses a high sampling efficiency, a fair degree of portability, and one that functions well at both low and high dust concentrations. The Owens dust sampling apparatus is very well suited to low dust concentrations, the konimeter for more intense conditions, while the electrostatic precipitator and the impinger are satisfactory for both low and high industrial dust concentrations. All of the instruments vary to some degree in their dust catching ability, with the degree of dustiness. It is for this reason that one is hardly justified in attempting to decide on the exact degree of dustiness in a given atmosphere. It must for the time being suffice reasonably to approximate the degree of dustiness by

means of the instrument best suited to the particular atmosphere under study. For a more complete discussion of the relative efficiencies of certain of these instruments the reader is referred to studies conducted by the U. S. Public Health Service, Bureau of Mines, and certain other organizations.[12]

DUST AND PULMONARY DISEASE

That certain groups of persons are, by virtue of their occupation, exposed to excessive quantities of atmospheric dust and thereby suffer high rates of mortality from respiratory disease, was long ago noted by philosophers and students of industrial hygiene. Undoubtedly the earliest examples of exposure to hazardous dusts must have been in connection with implement making, mining, quarrying, pottery manufacture, and the building of homes by such persons as the cliff-dwellers.

There are early references to this subject and no less a person than Hippocrates has noted the effects of mining as a dusty occupation. He speaks of the metal digger as a man who "has his right hypochondrium bent, a large spleen, and a costive belly; breathes with difficulty, is of pale, wan complexion, and is liable to swellings in his left knee." Pliny the elder, writing in the first century is, perhaps, the first to mention the use of protective devices for workers in the dusty trades. He says:

"Those employed in the works preparing vermillion cover their faces with a bladder skin, that they may not inhale the pernicious powder, yet they can see through the skin." In more recent times Georges Agricola (1556), Ramazzini and Professor C. T. Thackrah (1822) have described the effects of the inhalation of dust on the health of the worker.

If one carefully examines curves for the death rate from pulmonary tuberculosis in the rural districts of the United States or Great Britain, one will find that the death rate for males and females will be very close together. In general the male rate will be found to be very close to the female rate or somewhat less than the rate for females below the age of about 15 years. After this age the male rate is somewhat greater than the female rate but in general they are rather in agreement. Contrasted with this are the curves for the death rate in urban districts. In this case the death rate for males over the age of fifteen years is greatly in excess of that for females. Public health authorities agree that the industrialism of city life is largely responsible for this difference causing the rate for males to be in excess. This is a rather gross presentation of the facts and certainly a further analysis is warranted. The difficulty of the situation arises because it is very difficult to secure accurate mortality data for groups of persons exposed to various dusts. In view of this, workers in this field have had recourse to the use of ratio statistics. The ratio of the deaths from tuberculosis to the total deaths in any exposed group, may be used as an indication of the importance of tuberculosis as a causative factor of death exposed group. In the following tables are presented certain of these ratios taken from data presented by the United States Registration Census for the registration area and the Prudential Life Insurance Company.

Comparing specific trades in the Prudential experience with the Prudential group as a whole, and comparing specific trades in the registration area with

the registration group as a whole, both sets of data are highly illuminating and bring out very clearly the excessive tuberculosis ratios characteristic of certain occupations. The two sets of figures, allowing for the constantly higher ratios throughout the Prudential experience, check each other very closely, even in such details as the concentration of the highest ratios at ages under 35 among jewelers, and at ages over 35 among marble and stone cutters. There must always be a large measure of doubt in regard to the significance of high tuberculosis ratios. Ratios, as distinct from rates, depend on two independent variables, and a high ratio of tuberculosis deaths to total deaths may be produced by a low mortality from other causes, as well as by a high mortality from tuberculosis.

TABLE 1. *Ratios, in Per Cent, of Tuberculosis Deaths to Total Deaths in Occupations Exposed to Mineral and Metallic Dusts.*[1]

Occupation Groups	United States Registration Area, Age Groups						Prudential Experience, Age Groups					
	15–24	25–34	35–44	45–54	55–64	15 and over	15–24	25–34	35–44	45–54	55–64	15 and over
All occupied males	28.1	30.9	24.0	14.4	7.6	14.9	33.2	40.9	32.9	19.0	8.8	20.5
Brick, tile, and terra cotta workers	...	...	...	...	...	...	22.9	35.3	19.8	18.6	10.7	15.6
Iron and steel workers	19.8	26.1	23.3	16.7	8.5	16.9	30.0	34.1	31.3	14.7	8.7	21.0
Plasterers	25.0	31.5	34.5	16.4	7.8	16.7	34.5	43.6	40.4	23.5	11.8	21.9
Molders	...	...	...	...	...	...	23.7	40.4	30.7	21.6	13.9	23.0
Paper hangers	...	...	...	...	...	...	35.1	44.0	42.5	15.7	11.5	29.1
Painters, glaziers, and varnishers	30.8	36.9	29.2	17.4	9.0	18.7	...	...	...	...	...	...
Tinplate and tinware workers	39.4	36.7	34.8	13.7	6.6	18.7	...	...	...	...	...	...
Jewelers	50.0	39.7	23.4	14.1	8.5	17.8	50.9	58.3	45.3	21.2	11.1	29.3
Glassblowers	...	...	...	...	...	...	45.1	53.3	31.3	28.3	15.4	32.1
Other glassworkers	...	...	...	...	...	...	31.5	51.1	34.4	23.1	15.5	30.5
Glassworkers	47.2	42.6	33.1	19.7	7.9	30.0	...	...	...	...	...	...
Tool and instrument makers	...	...	...	...	...	...	37.5	52.7	36.9	33.7	10.4	31.9
Potters	...	...	...	...	...	...	31.2	49.6	39.8	30.2	21.1	32.2
Marble and stone cutters	26.2	43.5	44.1	41.6	23.3	30.7	38.3	51.1	44.4	39.0	26.7	33.6
Brassworkers	...	...	...	...	...	...	58.2	51.0	43.8	24.2	16.1	36.7
Compositors and typesetters	...	...	...	...	...	...	46.3	55.9	41.1	24.9	9.8	36.8
Pressmen	...	...	...	...	...	...	42.9	47.7	44.0	20.0	11.1	39.6
Printers, lithographers, and pressmen	43.6	50.0	36.3	21.5	7.7	29.5	...	...	...	...	...	...
Polishers	...	...	...	...	...	...	43.4	56.1	44.0	24.9	14.3	36.8

[1] The figures given are taken from Bulletin No. 231, Bureau of Labor Statistics, United States Department of Labor.

STUDIES OF THE ACTUAL DEATH RATES FROM TUBERCULOSIS IN VARIOUS DUSTY TRADES

It is by no means intended to discredit all use of mortality ratios, and still less to throw doubt upon the real importance of the problem of industrial tuberculosis. We desire only to emphasize the possible fallacies in the use of ratios, and the necessity for controlling deductions by the determination of actual death rates wherever possible.

In England numerous statistics are available which indicate that in many industrial employments, such as metal mining, marble and stone cutting, and grinding and polishing, high tuberculosis ratios are associated with high tuberculosis rates. In Table 2, for example, are presented the data for occupations exposed to metallic dusts, from the Sixty-Fifth Annual Report of the Registrar-General, rearranged and supplemented by ratio computations. They show that the excessive ratios of tuberculosis deaths to total deaths, indicate an actual excess death rate from tuberculosis of one to two persons per thousand population; while at the later age periods, the death rate from causes other than tuberculosis among the workers exposed to the influence of metallic dusts, is also well above the normal rate.

TABLE 2. *Mortality from Pulmonary Tuberculosis and Other Causes in Occupations Exposed to Metallic Dust, Compared with that of All Occupied Males, England and Wales, 1900–1902.* *

Age Period	All Occupied Males				Occupations Exposed to Metallic Dusts			
	Deaths per 1,000			Per Cent Due to Tuber- culosis	Deaths per 1,000			Per Cent Due to Tuber- culosis
	Total	Tuber- culosis	Other Causes		Total	Tuber- culosis	Other Causes	
15–19	2.4	0 5	1 9	22	2.7	0 7	2 0	27
20–24	4.4	1.5	2 9	35	5 3	2.7	2 6	52
25–34	6.0	2.0	4 0	34	6 3	3 3	3 0	53
35–44	10 2	2.7	7 5	27	11 7	5 0	6 7	43
45–54	17 7	3 0	14 7	17	21.0	5 2	15 8	25
55–64	31.0	2 2	28 8	7	36 0	3 9	32 1	11
65 and over........	88 4	1.1	87 3	1	95 5	1.5	94 0	2

* Bulletin 231, U. S. Bureau of Statistics.

The earlier reports of the medical officer of health of the city of Sheffield contain particularly significant data in regard to the mortality in the intensively hazardous processes of cutlery industry. The report for 1910, for example, shows a mortality from pulmonary tuberculosis among grinders of 14.8 per thousand for the age of 18 and over, as compared with a rate of 2.7 for all occupied males of the age of 20 and over. The corresponding mortality from all other causes was 15.1 per thousand for grinders and 13.7 for all occupied males; the ratio of tuberculosis deaths to total deaths was 49 per cent for grinders and 16 per cent for all occupied males

In the United States we have a few- but only a very few— data of this kind which show the actual death rates from tuberculosis in employments exposed to the hazards of industrial dusts. The only general collection of statistics of this sort with which we are familiar, was presented in a special bulletin on "Tuberculosis in the United States," prepared by the Bureau of the Census for the meeting of the International Congress of Tuberculosis, held in Washington in 1908. The highest and the lowest rates included in this tabulation are presented in Table 3, and they are suggestive and interesting, although the absence of an analysis by age periods detracts seriously from the value of the results, as does the fact that the occupational groups are large and often loosely defined. The high rates among cigarmakers and tobacco workers,

Table 3. *Mortality from Tuberculosis in Certain Occupations in the Registration States for the Age of 10 Years and Over, 1900.*

Occupation	Deaths per 100,000	Occupation	Deaths per 100,000
Marble and stone cutters........	540.5	Steam-railroad employees	129.8
Cigarmakers and tobacco workers	476.9	Clergymen	123.5
Compositors, printers, and press-		Miners and quarrymen..........	120.9
men	435.9	Farmers, planters, and farm la-	
Servants	430.3	borers	111.7
Bookkeepers, clerks, and copyists	398.0	Lumbermen and raftsmen........	107.1
Laborers (not agricultural)......	370.0	Bankers, brokers, and officials of	
All occupied males.............	236.7	companies	92.1

* Bulletin No. 231, U. S. Bureau of Statistics.

compositors, printers and pressmen, servants, bookkeepers, clerks and copyists, and the low rates among bankers, brokers, and officials of companies are no doubt in large measure due to the age composition of the respective groups. In the high rate among servants the racial factor must certainly play an important part.

Important data in regard to the effect of mineral dusts upon the tuberculosis rate have recently been presented in the "Second Preliminary Report of the Committee on Mortality from Tuberculosis in Dusty Trades",[13] which deals primarily with conditions in the quarrying districts of Vermont. We have presented in Table 4 certain selected data from this report which indicate that in towns, and even in entire districts, where a considerable proportion of the population is exposed to mineral dust, the tuberculosis death rate for the entire administrative unit may be increased far above the normal value.

Table 4. *Mortality from Tuberculosis of the Lungs in Quarrying Districts of Vermont, 1906–1915.*

District	Tuberculosis Death Rate per 100,000 Population	District	Tuberculosis Death Rate per 100,000 Population
State of Vermont...............	90.6	Town of Dorset (marble center)	149.4
Granite-cutting districts	143.0	Slate districts	111.3
Barre City (granite center)......	233.2	Town of Castleton (slate center)	176.0
Marble districts	97.1		

* Bulletin 231, U. S. Bureau of Statistics.

A comprehensive study of this kind conducted by Drury deals with the incidence of tuberculosis among the employees of an ax factory in the State of Connecticut. The factory in question employs about 800 men and is situated in a rural community where other industrial activities are largely agricultural. The vital statistics for the four adjacent towns in which the

[13] Second Preliminary Report of the Committee on Mortality from Tuberculosis in Dusty Trades. National Tuberculosis Ass'n, New York (1919).

operatives might reside have been analyzed in detail for a period of 20 years, and each death certificate for tuberculosis or other respiratory disease has been transcribed and investigated. The fact that the medical consultant of the ax factory and the superintendent had both been in the employ of the company during the two decades covered by the investigation, made it possible to trace out practically every death certificate, and to determine the actual occupation of the deceased. The final analysis of the results yielded the astonishing figures presented in Table 5.

Thus, we find the entire population of the mill district showing a tuberculosis rate of 200, as compared with 150 for the State as a whole. The mill population itself has a rate rising to 650, and the group of polishers and grinders the astounding rate of 1,900. The other employees of the mill are not entirely comparable in age, race and general social and economic status with the polishers and grinders, but it is evident that the high death rate among the polishers and grinders, who suffer from a tuberculosis death rate over ten times the normal rate, is primarily due to the hazards of their occupation. Nor is tuberculosis the only form in which they pay a penalty for their hazardous employment. Dr. Drury reports that the mortality from pulmonary infections other than tuberculosis for the period 1900 to 1919 was 430 per 100,000 for the polishers and grinders, as compared with 170 for the other employees of the ax factory.

TABLE 5. *Mortality from Tuberculosis of the Lungs in a Connecticut Ax Factory, 1900–1919.*

District or Group	Death Rate per 100,000	District or Group	Death Rate per 100,000
State of Connecticut	150	Employees of ax factory (all)	650
State of Connecticut (male population)	170	Employees of ax factory, polishers and grinders	1,900
Ax factory district (3 towns, entire population)	200	Employees of ax factory, others	160

Mention must be made of a recent and intensive study of this subject by Dr. F. L. Hoffman,[14] among the granite cutters of Washington and Caledonia Counties of the State of Vermont. This study, in which the mortality data for 26 years were analyzed, included the study of 18,406 death certificates; sanitary surveys were made of the homes and working places of the groups studied, the anthropometric status of 1,869 persons engaged in the industry was determined, and lastly, intensive physical examinations and X-ray pictures of 427 of the men were made.

This study disclosed the fact that the granite-stone industry is carried on by wage earners who live under sanitary conditions above the average, whose housing conditions are above the average, and whose anthropometric status is indicative of a superior physique. Yet this group suffers a very high mortality for tuberculosis, as disclosed in Table 6.

Dr. Hoffman at one point tersely summarizes his conclusions as follows:

"The general conclusions derived from these data would therefore seem to support the theory that granite cutters in the State of Vermont are subject

[14] Hoffman, *U. S. Bur. Labor Statistics Bulletin* **293**, Washington (1922).

TABLE 6. *Mortality from Pulmonary Tuberculosis Among Granite Cutters, Compared with that of the General Adult Population of Vermont, 1896–1918, by 5-Year Periods.*

(Data for granite cutters taken from experience of the Granite Cutters' International Association.)

	Granite Cutters				General Adult Population (20 Years of Age and Over)	
Period	Number Exposed	Deaths	Death Rate per 100,000	Aggregate Population	Deaths	Death Rate per 100,000
1896-1899	5,584	22	394.0	862,468	1,636	189.7
1900-1904	10,747	38	353.6	1,099,708	1.821	165.6
1905-1909	14,594	105	719.5	1,120,253	1,669	149.0
1910-1914	17,103	137	801.0	1,140,798	1,370	120.1
1915-1918[1]	12,494	133	1,064.5	694,341	752	108.3[2]

[1] Exclusive of last three months of 1918.
[2] 1915-1917.

to an excessive frequency of death from pulmonary tuberculosis, not because of an exceptional risk of contact infection, or because of inferior physique, or because of unfavorable housing conditions or other sanitary deficiencies, but primarily because of the occupational exposure, which in its final analysis is reduced to the dust hazard resulting from the excessive use of pneumatic tools."

THE SPECIFIC INFLUENCE OF PARTICULAR INDUSTRIAL DUSTS IN RELATION TO TUBERCULOSIS

It is an interesting and significant fact that in every instance, as far as we are aware, in which a heavy incidence of tuberculosis has been clearly shown to result from exposure to industrial dust, the dust in question has been in part at least made up of crystalline rock. It is silicosis which lies at the basis of miners' phthisis, and silicosis is probably the chief predisposing factor in tuberculosis among ax grinders, although in grinding and polishing, steel dust may, and probably does, play a part as well. No such striking statistical results as those cited for mining, quarrying, and grinding, and pottery making have yet been presented for industries where crystalline rock particles were not involved. But silica dust is not looked upon as the predisposing agent only in the cases where it is present in excessive quantities. It is, indeed, being pointed out as the harmful agent even in those cases where it is present in smaller and less significant amounts. Recently this very question has been under discussion. Dr. E. H. Ross, in an interesting letter in the London Daily Times quoted by Dr. Hoffman,[15] attributes the excessive mortality from phthisis among printers to silica dust present in the printers "list". It is to be remarked in passing, however, that in the minds of many workers in the field, the existence of quantities of silica dust sufficient to cause the excessive phthisis rate in the printing trades is not proved beyond doubt.[16, 17]

It may be well to present an interesting table taken largely from Teleky[18]

[15] Hoffman, *U. S. Bur. Labor Statistics, Monthly Labor Review,* 15, 179 (1922).
[16] Roos, *J. Ind. Hyg.,* 3, 257 (1922).
[17] Collis, Letter in London Daily Times, No. 42, 553, p. 8 (October 28, 1920).
[18] Teleky, "Arbeit und Gesundheit," Vol 7, Reimar Hobbling, Berlin (1928).

and Collis [19] showing the relation between the death rate from tuberculosis and the free silica content of the respired dust.

TABLE 7. *Mortality from Tuberculosis and the Free Silica Content of the Dust in Certain Specified Occupations.*

Occupation	Free Silica	Annual Death Rate per 1000 Living	Ratio Tuberculosis Deaths to Total Deaths
Flint knappers	100%	41.0	77.8
Ganister miners	95	22.3	67.8
Tin miners	75	17.6	42.0
Sandstone masons	Up to 95%	16.7	52.4
Grinders (Sheffield)	50–100	15.0	49.7
Granite cutters	30	5-11	47.8
Gold mining (Bendigo)....	Silica rock	12.7	23.5
Granite cutters (Aberdeen)...........	30	5.7	38.0

In general it will be observed that there exists a rather close relation between the death rate from tuberculosis and the quantity of free silica in the air breathed by the worker.

Turning now from the consideration of silica dust—of major importance in the problem of industrial tuberculosis—to other kinds of dusts, we find a strikingly different picture.

Thackrah points out that bricklayers, lime workers, and plasterers and whitewashers, all of whom are exposed to lime dust, suffer from it no sensible injury. Reckzeh investigated the relation of lime-dust inhalation to pulmonary tuberculosis, and found that tuberculosis was rare in lime-producing districts. Selkirk,[20] quoted in the Journal of the American Medical Association also found phthisis to be rare among lime workers. He is so firm in his belief as to the beneficial effects of lime dust, that he even hints at the organization of lime works as curative tuberculosis colonies. Recent work in Japan has confirmed the findings of Reckzeh and Selkirk: Nagai [21] found tuberculosis to be uncommon among workers about lime kilns, and also that lime dust had no detrimental effect on the lung tissue of guinea pigs subjected to it.

Ample evidence is at hand to show that the inhalation of cement dust also does not predispose to tuberculosis. In this industry enormous amounts of dust are found in the grinding and sacking rooms, so that there exists no question as to definite dust exposure. Dr. G. E. Tucker [22] studied the problem at a cement mill in California. He concluded:

"As a result of an investigation of the dust problem in conjunction with the manufacture of Portland cement, based upon the review of literature on the subject, the examination of 956 employees in one plant, examinations of men employed in the dusty departments of four other mills, the medical records of employees and guinea pig experimentation, there appears to me to be no evidence of injurious effects from cement dust upon employees engaged in its manufacture."

[19] Collis, Milroy Lectures (1915).
[20] Selkirk, *Brit. Med. J.*, Nov. 14, 1908. Abstracted in *J. Am. Med. Assoc.*, 51, 2093 (1908).
[21] Nagai, Abstracted in *J. Ind. Hyg.*, Abstract Section, 1, 146 (1920).
[22] Tucker, *Am. J. Public Health*, 5, 560 (1915).

The clearest and most striking case, however, is that of coal dust, which, if it has any effect, appears to exert a protective influence against the development of active tuberculosis. A clearly marked fibrosis (anthracosis) follows the inhalation of coal dust, a condition which appears to favor a high mortality from acute respiratory disease; but the tuberculosis death rate among coal miners is uniformly and characteristically low. The figures presented in Table 8, below, from Dr. Hoffman's study [23] bring out the typical relation with clearness.

TABLE 8. *Mortality in Coal-Mining Districts of Pennsylvania.*

District	Death Rate per 100,000		
	Pulmonary Tuberculosis	Other Forms of Tuber- culosis	Other Respiratory Diseases
Scranton	79.9	16.6	261.2
Wilkes-Barré	74.9	19.4	212.5
Remainder of State	110.5	16.4	184.2

That coal miners suffer a high mortality from acute respiratory diseases has been noted by many workers in this field. Wainwright and Nichols,[24] Collis [19] and lastly, Dr. Dublin [25] have made this point very clear and practically incontrovertible.

Dr. Dublin, in his paper, presents the following very interesting table:

TABLE 9. *Influenza-Pneumonia Mortality Among Bituminous Coal Miners, October to December, 1918, Compared with All Occupied White Males, Industrial Department, Metropolitan Life Insurance Co.*

Age Period	Annual Death Rate per 1000	
	Bituminous Coal Miners	All Industrial White Males
All ages	50 1	22.3
15–25	29 5	17.5
25–45	62.1	32.6
45–65	44.4	11.7

And lastly, the occupational statistics presented by the Registrar-General of Great Britain include a particularly striking comparison between coal miners and tin miners, the former exposed to coal dust, the latter to hard crystalline dust. The comparative mortality figures for tuberculosis in 1900-1902 were 186 for all occupied males, 85 for coal miners, and 838 for tin miners.

DUSTS AND INDUSTRIAL POISONING

In addition to the respiratory diseases caused by the inhalation of various irritating dusts there exists, in industry particularly, a large amount of poisoning produced by the inhalation of various toxic dusts. Foremost among the poisons, from the viewpoint of the number of cases, is lead. The poisonings

[23] Hoffman, *U. S. Bur. Labor Statistics Bull.* **231**, Washington, D. C. (1918).
[24] Wainwright and Nichols, *Am J. Med Sci.*, **130**, 403 (1905).
[25] Dublin, *J. Ind. Hygiene*, **1**, 483 (1920).

in industry due to lead dust arise chiefly in the manufacture of basic lead carbonate for use in paints, lead oxides used in storage battery manufacture, lead sulphate used in rubber compounding, lead chromate used in paint manufacture, and lead sulphide encountered chiefly in the mining industry, in the printing trades and in the painting occupations, particularly in the latter case when associated with dry sandpapering of lead painted surfaces.

The route of absorption of lead is largely, in fact practically completely, by way of the lungs. It is true that some lead dust may be and is swallowed, but as we shall see later this may be directly excreted with little or no real absorption, and hence produce little or no damage.

Due chiefly to the work of Aub [26] and his colleagues at the Harvard Medical School, the mechanism of lead poisoning is now quite clear. Lead may be taken into the body by three routes, ingestion, inhalation, and absorption through the skin. The latter method is an uncommon one, in fact only in certain cases such as, for example, with lead tetraethyl, does absorption take place in this manner. For the purposes of our discussion, we may limit ourselves to the more important methods of ingestion and inhalation. When lead is ingested some (in the case of dusts insoluble in the gastric juices this may constitute a major portion) passes through the intestinal tract and is directly eliminated in the feces. In fact, by far the greatest portion of ingested lead is eliminated in this manner. A portion may, however, be dissolved in the gastric juice, absorbed from the intestinal canal by the blood stream and stored in the body or eliminated in the urine. In the case of inhaled lead, the result is far different. Here, practically all of the lead passes into the systemic circulation and is stored in the body or eliminated through the kidneys in the urine. Between the point of absorption (that is, the lungs) and the elimination via the kidneys the lead is held in the blood stream and in the body tissues. The Harvard studies clearly show that considerable quantities of lead may be stored in the liver, but by far the largest amount of lead is stored in the skeletal bones of the body. The amount of lead that may be found in the liver depends on the route of absorption, if this has taken place through the lungs a lesser amount will be found therein. So far as known at the present time, the actual existence of lead in the bones of the body is not accountable for any of the symptoms of lead poisoning. It is only when the lead is released from the bones and invades the body tissues that symptoms begin to manifest themselves.

It appears that the lead stored in the bones is in a chemical state, somewhat analogous to that of calcium. A diet low in calcium, for example, will serve to release lead from the bones into the systemic circulation and tissues; so also will diets containing acids, acid forming salts and alkalies. The combination of a diet low in calcium and rich in acid-forming salts, is an ideal one for increasing the discharge of lead from the bones and its elimination in the urine. It is possible with this newer knowledge concerning the chemistry of lead poisoning, to accelerate the removal of lead from the body tissues and hasten its deposition in the bones. After this is accomplished, the lead may be slowly removed from the bones and eliminated from the body by the feeding of diets low in calcium, as outlined above.

The composition as well as the concentration of the lead compounds to which the worker is exposed, plays an important rôle in determining the time of onset of disease, for it is the composition which determines the solubility

[26] Aub, Fairhall, Minot, Reznikoff, Medicine Monographs, No. 7, Baltimore (1926).

tries where there was a severe lead hazard (Group E) and for the periods of exposures studied was not sufficient to produce detectable symptoms of lead poisoning." On the other hand drivers of cars using ethyl gasoline as a fuel for two years showed no definite signs of lead absorption. This committee decided that no good reason existed for prohibiting the use of ethyl gasoline provided the distribution and use are controlled by proper regulations.

Largely as a result of these two studies the sale and use of ethyl gasoline has been permitted in the United States. There are investigators in this country who still do not feel that the question is completely closed. Hamilton, Reznikoff and Burnham [31c] analysed the report of the Bureau of Mines and it was "found to be inadequate in scope, in technic and conclusiveness." Lately a warning has been issued by one of America's leading physiologists, Dr. A. J. Carlson of the University of Chicago.[31d] He says, "Pollution of air and water by modern cities and modern industry is on the increase, and the probable danger from these conditions seems only partly appreciated by society. Formerly, lead poisoning was largely confined to workers in lead industries. Now lead is blown into the air from the exhaust of nearly every automobile. Lead and arsenic are taken into our system with the apple and the pear. We may not inhale enough lead in our breathing or consume enough lead and arsenic in our fruit to produce acute poisoning and tissue injury, but who is there to say that this slow assimilation of metallic poisons brought about by modern industry is without danger and ultimate injury? The only factor of safety that I can see in this situation is the phenomenon of tolerance, that is, the capacity of the living organism, if it is not seriously wrecked by the poisons, to so adjust the internal processes as to render the poisons less and less injurious. Tolerance means physiologic compromise, but such continued existence does not necessarily mean the higher life. Continued increase of population means increased growth of cities, increased industrial concentration and increased industrial poisoning, despite all measures to eliminate the latter."

The Surgeon General's committee was itself cognizant of the fact that their conclusions were based on a small experience of but a rather limited duration. The committee in closing its report says: "It remains possible that, if the use of leaded gasolines becomes widespread, conditions may arise very different from those studied by us which would render its use more of a hazard than would appear to be the case from this investigation. Longer experience may show that even such slight storage of lead as was observed in these studies may lead eventually in susceptible individuals to a recognizable lead poisoning or to chronic degenerative diseases of a less obvious character. In view of such possibilities the committee feels that the investigation begun under their direction must not be allowed to lapse."

NON-POISONOUS DUSTS WHICH PRODUCE CONSTITUTIONAL SYMPTOMS

So many of the industrial poisons are dusts that one cannot devote space to a discussion of them all. We have already considered the case of lead poisoning which may serve as an example of the part played by dust in the causation of one of the most important of the industrial poisons. Another very interesting substance from our point of view is zinc. This metal is not

[31c] Hamilton, Reznikoff and Burnham, *J. Am. Med. Assoc.*, **84**, 1481 (1825).
[31d] Carlson, A. J., *Science*, N. S., **67**, 355 (1928).

"per se" poisonous or toxic.[32] In certain industries such as the making and casting of brass, the metal is carried to a temperature above that of the vaporization point of the zinc, with the result that dense zinc oxide fumes are given off. The inhalation of this finely divided zinc gives rise to certain symptoms in the workers, characterized by chills and usually referred to as brass casters chills or "shakes". For a long time these chills were associated only with the casting of brass and in brass founding, but now it is generally accepted that they are produced in industry by cold zinc oxide, such as in the bag house of the plant studied by Batchelor, et al., in the study previously cited.[32] That these typical symptoms may be produced experimentally, is clearly shown by the observations of Sturgis, Drinker and Thomson; [33] they caused zinc oxide to be inhaled by two men in whom typical "chills" took place. It is interesting to note that many other metals in addition to zinc may produce "chills" or "shakes", according to Koelsch,[34] and Drinker, Thomson and Finn [35] have recently shown that magnesium oxide fume may cause "chills" when inhaled. The cause of the characteristic chills of metal fume inhalation is not known. It is believed by some workers that the dryness and the peculiarly penetrating power of these fumes makes it possible for them to bring about this characteristic effect. It is well known in the case of zinc and magnesium that the "chill" is accompanied by a leucocytosis, and that this is the only objective finding associated with the attack."[33]

It is interesting and important to note that this phenomenon represents a peculiar and characteristic result of the inhalation of an aerosol composed of finely divided metal. It seems hardly necessary to add that there exists in certain persons a peculiar heightened susceptibility to the inhalation of certain pollens and certain other organic dusts. Common amongst these is the pollen of the giant ragweed, rose, and the dusts of hair, feathers, silk, etc. Recently the dust produced by the dry residue of the castor oil bean has been shown to be the cause of asthmatic attacks among persons living in the vicinity of the factory in which this material was pulverized.[36]

THE PROBLEM OF ATMOSPHERIC SMOKE POLLUTION

Of late much attention is being given to the problem of smoke and its relation, both directly and indirectly, to health. This is pertinent to our problem, for smoke is an aerosol composed of particles of liquid or tarry material, solid matter and gases produced by the combustion of certain fuels.

The effects of smoke may well be grouped in two categories. These are, firstly, the direct effects of smoke, such as those produced by the inhalation of the solid and tarry materials; and, secondly, the indirect effects, those due to the loss of certain rays of the sun's light, such as the ultra violet rays, or the direct sunshine which is so stimulating and invigorating. And finally, mention must be made of the changes brought about in the climatic conditions of certain localities due to the presence of large amounts of smoke and the consequent haze or fog and rain produced partly thereby.

[32] Batchelor, Fehnel, Thomson, R. T., and Drinker, K. R., "A Clinical and Laboratory Study of the Effect of Metallic Zinc, Zinc Oxide, etc.," *J. Ind. Hyg.*, **8**, 322 (1926).
[33] Sturgis, Drinker and Thomson, *J. Ind. Hyg.*, **9**, 88 (1927).
[34] Koelsch, *J. Ind. Hyg.*, **5**, 87 (1923-24).
[35] Drinker, Thomson and Finn, *J. Ind. Hyg.*, **9**, 187 (1927).
[36] Figley and Elrod, *J. Am. Med. Assoc.*, **92**, 79 (1928).

The Extent and Nature of Atmospheric Pollution

Studies of the extent of atmospheric pollution have been made in the United States by Whipple and Whipple,[37] who suspended water pails high above the street level for the purpose of catching the soot and dustfall, and further studies are recorded in the smoke investigations of the Mellon Institute.[38] But by far the most satisfactory work along these lines is that of the Advisory Committee on Atmospheric Pollution of the British Meteorological Office. These latter studies started in 1913 represent an excellent scientific study of the problem of the nature and extent of atmospheric pollution. These studies consisted in researches for the development of new methods for the determination of the relation between impurities and visibility (a photometric method) and an apparatus for the determination of the amount of water in the form of droplets. In addition, the amount of impurity falling on a standard area was determined. By means of a continuous automatic filter, two to four filtration samples per hour are made on a piece of filter paper, thus providing an automatic record for examining the changes in the condition of the atmosphere from time to time throughout the year; and lastly, the jet dust counter

TABLE 13. *The Soot-Fall in Certain Urban and Rural Areas.*

Locality	Soot-fall (Tons per Sq. Mile per Year)
Leeds:	
Industrial center	539
Suburban area	26
London:	
Center of city	426
Average for whole metropolitan area	248
Glasgow: center	820
Pittsburgh	1,031

was used which yielded valuable records of the actual dust content of the air and the shape and nature of the particles.

The most complete studies indicate that the amount of atmospheric pollu-

TABLE 14. *Soot-fall, London, England—Meteorological Office Station (1922–1923).*

	Solids in Metric Tons per Hundred Square Kilometers
Insoluble matter	
Tar	295
Carbonaceous other than tar	2,509
Ash	5,360
Soluble matter	
Loss on ignition	1,528
Ash	3,321
Total solids	13,013
Included in soluble matter	
Sulfates	1,125
Chlorine	1,128
Ammonia	131

[37] Whipple and Whipple, *Trans. Am. Soc. Heating Vent. Engrs.*, **21**, 211 (1915).
[38] "Papers on the Influence of Smoke on Health," Bulletin No. 9, Smoke Investigation, University of Pittsburgh (1914).

tion in urban areas as measured by soot-fall varies from between 200 to approximately 1,000 tons per square mile per year. The following table reproduced from Gibbs [39] brings out the facts with clarity and shows the striking difference between a suburban and an industrial center.

It may be of interest to present the data (Table 14) taken from the 1923 Report of the British Committee on Atmospheric Pollution. The data presented are for the year 1922-23.

From these data one may obtain a picture of the general nature and extent of atmospheric pollution. It is easy to realize in the light of these findings that the combustion of fuels produces a condition in the urban areas which is quite different from that existing in the country. The coal smoke and soot pictured above may be avoided in a very large measure by the use of proper fuels and the practice of scientific methods of firing the boilers and heating devices. In order to achieve this end, it is only necessary to add the fuel to the fire box in such a manner that there always remains a sufficient area of hot coals to burn the gases and vapors coming from the fresh or "green" coal prior to its being completely coked. With this precaution, and certain other more minor ones which need not be discussed here, it is possible in a large measure to avoid the production of excessive quantities of smoke.[40, 41] That the combustion of fuels in an improper manner is largely the cause of this enormous soot-fall, is indicated by the changes in deposit noted after the campaign for proper firing in Pittsburgh, Pennsylvania, and in the differences in deposit obtained on week days as contrasted with Sundays and normal times with those of the famous British Coal Strike of 1921 when minimal quantities of coal were consumed.

The Direct Effects of Smoke on Health

Attempts have been made to measure the effect of smoke on health. We are told, for example, that Pittsburgh has an exceedingly high death rate from pneumonia and that this is brought about by the inhalation of smoke and combustion products. And contrasted with this high pneumonia rate Pittsburgh has a very low death rate from tuberculosis. The studies by the Pittsburgh smoke investigation committee have attempted to elucidate this relation. In one of the papers [38] presented by these workers, the smoke content of the air of Pittsburgh by wards is compared with the death rate from pneumonia. There appears to be a close relation between the two. The data, however, are very crude, that is, they have not been refined for age, sex, nationality, social and economic status, and hence it seems unfair to draw conclusions of too broad a character. The authors of this paper are themselves disturbed by the fact that Chicago, with little smoke in the air, has a very high pneumonia rate. Unpublished data of Brundage of the United States Public Health Service, disclosed the fact that the pneumonia rate among iron and steel workers is approximately twice that among employees in other industries for the three year period 1920-23. These data cover an experience of some 26,000 employees in the steel and iron industries and 55,000 in other industries. While it is true that iron and steel workers are usually housed in communities which have a relatively high degree of atmospheric pollution, this holds true

[39] Gibbs, "Clouds & Smokes," Blakiston, Philadelphia (1924). See also his paper in Vol. I of this series.
[40] Landvoight, J. Am. So. Heating Vent Engrs., 33, 607 (1927).
[41] Azle, Ibid., 33, 659 (1927).

in a large measure for all industrial groups, and Mr. Brundage's results indicate what is known from other studies, that the industrial environment has a very direct bearing on health. This factor was completely omitted from the Pittsburgh studies.

The British studies along these lines have been of this same type, in which the decrease in the death rate has been correlated with the decrease in soot-fall.

In conclusion, it is fair to say that while it seems reasonable to believe that there exists a relation between smoke and health, yet, so far as is known to the writer, this relation has not yet been proved to exist by the statistical material at hand.

The Indirect Effects of Smoke on Health

Invariably accompanying smoke at the time of its ejection from chimneys, there is a certain amount of sulfur dioxide and carbon monoxide (also due to automobiles). Carbon monoxide in outdoor air is rarely in excess of 1.0 part per 10,000 and sulfur dioxide rarely exceeds 0.2 part per million. In general, it may be said that carbon monoxide in this concentration represents no real hazard to health. The sulfur dioxide probably has no direct influence on health, but it is interesting to note that it may coat the particles of dust with a film of sulfuric acid. The presence of such hygroscopic particles promotes the formation of fog in air only moderately saturated with water vapor. In this way coal smoke produces fog of a very stable form. Smoke, therefore, may be said to promote fog formation, and by this the sunlight is occluded. In addition to this, smoke by itself (without fog production) may decrease sunlight by as much as 40-50 per cent. The British data indicate that this deprivation may even be considerably greater. While the total sunlight may be occluded to this extent the ultra-violet component is cut down to a proportionately greater extent on account of its shorter wave length.

We depend on the sun for warmth, light, and, as has only recently been shown, certain healthful rays, the ultra-violet rays which play such an important rôle in curing of rickets. The problem at hand reduces itself to the question of whether or not the deprivation which takes place really has an important bearing on health. According to Coblentz of the United States Bureau of Standards, the maximum ultra-violet radiation at sea level amounts to 3 per cent of the total incoming rays, and only between the hours of 9 A.M. and 3 P.M. in the winter months is the ultra-violet component of the sunlight of sufficient amount to be of possible therapeutic significance. That smoke may obscure these rays is clearly shown by the studies of the Medical Research Council of Great Britain.

That the lack of the ultra-violet rays, in amount such as might be obtained in winter without smoke occlusion, gives rise to disease still remains to be proven. It may, for example, be that the ultra-violet light absorbed in the summer time is sufficient as a rule to last the whole year. In spite of this lack of knowledge it would seem reasonable to make efforts to reduce this atmospheric pollution in an effort to take every possible safeguard with respect to life.

In closing, it should be mentioned that the production of fog depends on the proper degree of saturation of the atmosphere plus the existence of condensation nuclei, and as shown earlier particles coated with sulfuric acid make

an exceedingly stable fog. Fog in all probability has a direct effect on health in that at lower air temperatures it increases the rate of heat loss from the animal body, and may do this at so rapid a rate as to produce chilling effects. This indirect effect of smoke may have an important bearing on the health of the urban community—especially in such a climate as that of Great Britain.

Colloid Chemistry and Tuberculosis *

By Albert Mary,†

Founder of the Institut de Biophysique de Paris, Paris, France

On approaching the study of the pathology of tuberculosis, we are at once struck by the exclusive importance attributed to infection phenomena, and think we can explain the malady by a more or less detailed monograph on the *tuberculosis bacillus.* That, however, would be an unwise generalization, based on what happens in fermentable liquids. A particle of *Saccharomyces,* carried by the atmosphere, falls into a vessel containing a saccharine solution; there it feeds and multiplies, decomposing the sugar into alcohol and carbonic acid; the saccharine solution is changed, *"sick,"* ‡ because an organism is growing in it. A *tuberculosis bacillus,* carried by the air or by food, enters the respiratory or the digestive tract of a healthy organism; there it forms colonies, decomposes body fluids, and irritates the tissues in which it is implanted; the organism is tuberculous because the bacillus is living within it. This reasoning by analogy is very simple, and apparently so logical that all other considerations seem incidental. It leads us to picture the sickness as something extrinsic and *abstract,* as if it did not consist of a particular condition of a subject, but rather as if it were an entity instead of a modality, as if we could have a sickness without having a sick organism !

In reality tuberculosis, and especially pulmonary tuberculosis, is more than a mere chapter in bacteriology. Experimental tuberculosis has been obtained without the intervention of a single microbe. There is also spontaneous, *non-bacillary* tuberculosis, for instance, the inflammatory tuberculosis described by A. Poncet. Finally in the etiology even of bacillary tuberculosis, the primary pathological changes are not directly consequent on infection, but involve constitutional or accidental organic insufficiencies, leading to a profound modification of metabolism. I will not dilate here upon the dyspepsia and hepatic congestion referred to by many authors, and above all by Paul Carton,[1] Pascal Serph and Albert and Alexander Mary,[2] Pruvost.[3] The intervention of the acid-fast bacillus of Koch, when it is present, is delayed; this will be better understood when we explain that the specific effects of the bacillus are the result of a morbid evolution.

Phylogeny of Koch's Bacillus and Adsorption Phenomena

Acid resistance is not a *primitive* characteristic of the *tuberculosis bacillus.* Whoever has made cultures in glycerinated bouillon knows very well that the young bacteria are never acid-fast, which indicates that the bacillus discovered

* Translated by Jerome Alexander.
† Died Feb. 28, 1928.
‡ "Sarcina sickness" is a term well known in the brewing industry.   *J. A.*
[1] Carton, P., "La tuberculose par arthritisme." Paris, 1911.
[2] Serph, Pascal; Mary, Albert and Alexander, "De la cholemie consideree comme cause de la tuberculose," in *Le Médecin,* 1913, "Le rôle intoxicant de la bile," *ibid ,* 1913. "Esthésiometrie clinique," *ibid.,* 1913.
[3] Pruvost, Société de Médecine de Paris, 1919.

by Robert Koch comes from a bacterial species which is not acid-fast. Furthermore, those who regularly have charge of whole culture series of tubercle bacilli, have had occasion to observe that the flasks become turbid spontaneously and homogeneously without any contamination having occurred; J. Ferrán,[4] and following him S. Arloing,[5] P. Courmont, Auclair, Zupnik, Ravetllat, Garciá and Stephen Maher, have confirmed by numerous experiments the accidental or voluntary production of homogeneous cultures of mobile bacilli derived from the type of Koch. Immobility and a capsulated form are, therefore, no more basic characteristic of the *bacillus tuberculosis*, than is acid-fastness; for it can lose them and consequently must not have them. at the beginning of its evolution.   Jaime Ferrán has gone still further; he has identified four transition species. The X species exhibits all the characteristics of a common saprophyte of the *Coli* group, growing quickly and readily in almost any medium; the β species loses its ease of cultivation, and contains fatty substances which are not acid-fast; the λ species (Koch's acid-fast bacillus) contains lipoids and fatty acids, is capsulated and grows slowly in a special media; the δ species is a retrogressive type, slightly if at all acid-fast, mobile and saprophytic (homogeneous cultures of Ferrán, Arloing and P. Courmont).

What influences govern this bacterial evolution? Ferrán speaks of unexplained "jumps," of "mutations" in the sense of H. de Vries. But as Jerome Alexander [6] judiciously puts it, the mutations of organized beings are due to modifications of the living colloids by their physico-chemical environment. The bodies of bacteria are formed of colloidal granulations, and their biological changes involve the phenomena of colloidal fixation, of *adsorption*. Acid-fastness, for example, is in the nature of a retention, by the bacterial body, of lipoids and fatty acids. The micells of egg albumin, of silicates or of metallic ferrocyanides, acquire acid-fastness by a more or less prolonged maceration in sterile chicken bile. The same obtains for bacilli of the Coli group. The *bacillus coli vulgaris*, if cultivated in bouillon containing lecithin, begins to exhibit acid-fast properties after a dozen transplantations; resistance to decolorization becomes marked in old cultures.[7]

The microbe owes not only its color reaction to the "medium," but also its toxicity. It adsorbs not merely fatty acids (necrosis-producing, according to Camus and Pagniez,[8] but a number of organic products already prepared and circulating in the organism, such as hypoxanthine (Camura), and biuret substances, of which Ostrowsky says: "Peptones and albuminous products *fixed* by the bacterial body, increase its toxicity," [9] etc. . . . I might remark that lipoids readily form adsorption combinations with certain proteins; it is thus that Porges precipitation reaction is produced when a globulin comes into contact with lecithin.[10] Colloidal fixation thus explains the toxic evolution of corpuscles which are initially harmless; it traces back to the "soil" itself not only the receptivity but also and particularly the toxicity of microbes, a function of the adjacent intoxication.[11] Because of the adsorption compounds that the

[4] Ferrán, J., *Compt. rend*, 1897; "Ueber einige neue Entdeckungen bezueglich des bacillus der Tuberculose," *Wien. Klin. Woch*, 1898, "Travaux sur la nouvelle bactériologie de la Tuberculose," Barcelona, 1913
[5] Arloing, S., *Compt rend*, 1898.
[6] Alexander, Jerome, "Colloid Chemistry," 1924
[7] Mary, Albert and Alexander, "La acidoresistencia experimental," *Rev. Hygiene Tuberc.*, 1915.
[8] Camus and Pagniez, "Toxicité des acides gras," *Rev. sci*, 5, 698
[9] Dominici, H. and Ostrowski, E., "Recherches sur la poison du bacille de la tuberculose," Paris, 1914.
[10] Porges, *Berlin Med. Ges* 1907, Mary, Albert and Alexander, and Epailly, T., "Interprétation de la sero-réaction dans la syphilis," *Le Médecin*, 1913.
[11] Mary, Albert and Alexander, "Dictionnaire de Biologie physiciste," p. 385, Paris, 1921.

colloidal corpuscles of the bacterium's body form with the auto-toxins of the disease, the bacillus becomes, so to speak, a physico-chemical *condensation* of the primary intoxication. We can now understand the full biologic and pathologic significance of the masterly statement of Charrin: "Surely no one could emphasize more than I the great importance of bacteriology. . . . Nevertheless there are other things; there is, particularly, this essential doctrine of auto-intoxications, which develops parallel with bacteriology. . . . Perhaps we may even ask whether microbes, if placed in contact with a diastastic principle, may not, as to speak, incorporate this principle within themselves, like fibrin, in the experiments of Wuertz, seizes papaïn which remains fixed in it; after which, this filament of fibrin acts like this ferment." [12]

The essential fact in the etiology of tuberculosis being an endogenous intoxication, what is the nature of this intoxication?

THE ACIDOSIS OF TUBERCULOSIS

For ten years I have made a large series of conscientious analyses of urines coming from the most diverse illnesses. For this reason I can epitomize the nature of the urine in the first and second stages. The volume is almost always increased, the acidity uniformly diminished, mineral substances in excess, albumin frequent. These are the undoubted signs of acid intoxication, clinically reinforced either by latent nephritis or by edemas variously localized, wandering, transient.[13] The chemical nature of the acids that contribute to this intoxication, has up to the present been the object of sporadic investigations. Serph, my brother and I have demonstrated in the expectoration of the tuberculous, the presence of partially volatile fatty acids,[14] among which are probably biliary acids, and in the volatile group, small quantities of acetic, formic, and butyric acids. Oxalic acid is, in a more certain manner, one of the factors in tuberculous acidosis. The urine of the patients always contains an appreciable quantity of it, and it is found in the expectoration; I have quoted the case of a patient in the sanatorium of La Cronche (Isère), whose sputum showed persistently in the microscope octahedral crystals of calcium oxalate analogous to those found in the sediment of certain pathologic urines.[15] It would be in order, in concluding, to take up tuberculous acetemia; but the question is not yet definitely outlined. At all events the titrable alkalinity of the blood, which is normally 3.20 grams of NaOH per liter, drops to 2.40 grams or less in the tuberculous.

This acidosis causes a slowing up of general metabolic processes, and paralyzes phagocytosis; it is to be interpreted, from the point of view of colloid chemistry, by two manifestations of the highest importance: (1) increase in the *flocculability* of the serum, (2) the creation of *tuberculous tissue*.

1. MODIFICATION OF THE COLLOIDAL PROPERTIES OF THE BLOOD

The stability of the dispersed phase of the colloidal system of the blood is markedly diminished in the tuberculous. There is a tendency to flocculation, often discernible on direct ultramicroscopic examination, and made evident by precipitation reaction. We may recall the simplified technique that

[13] Charrin, "Les Poisons des Tissues."
[15] Mary, Albert, "Les Horizons du Physicisme," p. 12, Paris, 1923
[14] Serph, P., Mary, A. and A , "Crachat tuberculeux," *Le Médecin*, 1913.
[16] Mary, Albert, "Química y bacteriologia del esputo tuberculoso," *Laboratorio*, 1918.

Porges and Meier have tried to substitute for the complex method of Wassermann in the sero-diagnosis of syphilis: these authors bring into contact, at room temperature, some of the diluted suspected serum with an emulsion of lecithin in physiological salt solution. After three or four hours, if the serum is syphilitic, there is a precipitation. There is nothing specific about this reaction (nor that of Wassermann). Though negative with normal serum, it is positive if we use the serum of a person with confirmed tuberculosis.[16]

The depression of the titratable alkalinity is not the only cause of the physical instability of the blood in tuberculosis. Other factors must be faced —first of all oxalemia. The observations above referred to indicate that oxalic acid circulates principally in the form of insoluble calcium oxalate, colloidal, however,** and it is known that contrary to the alkaline oxalates (soluble) which maintain the blood in fluid condition, calcium oxalate exercises a powerful thromboplastic action.[17] Then there is lipemia (opalescent serum). This is rather common in pathological cases arising from various acidoses (acute, sub-acute, and chronic nephritis), according to Widal and Sicard.[18] Furthermore is it not surprising that Jousset has observed a milkiness of the serum in acute tuberculosis, and also, though less frequently, in chronic pulmonary tuberculosis.[19] Now lipemia tends to break the colloidal equilibrium of the plasma, as may be demonstrated by experiments *in vitro:* the mixture of stable plasma with a fine emulsion of any fatty body (olive oil) suffices to occasion coagulation.[20] The blood of the tuberculous is so readily flocculated, that the prelude to the lesion often seems to be, in confirmation with the general hypothesis of A. Lumière, primarily an occlusion of the capillaries.[21] H. Dominici and E. Ostrovsky have produced typical tuberculous lesions in guinea-pigs by the injection of colloidal or diffusible toxins extracted from Koch's bacillus;[22] and in their remarkable specimens, there are to be seen only obliterated vascular units, hypertrophied venules and arterioles, dislocation of the vascular walls, various indications of the obliteration of the vessels, beginning with the capillaries. By this same embolic mechanism are determined "Mary's micellular pseudo-tuberculosis," to which I shall presently return. When the tuberculosis is in an advanced stage and there is no hope of a favorable termination, it seems that the blood serum tends to pass from a condition of hypoalkalinity to one of hyperalkalinity. I conducted two series of observations, one covering fifteen, the other eighteen months. Neither case was carried to a conclusion, although the first case was dropped only when there was pronounced cavitation. According to Roussy, the same inversion occurs in cancer. As may be seen from the course of the graphs (Fig. 1), the evolution to alkalinity of titration occurs by irregular "crises."

2. Creation of Tuberculous Tissue·

When we speak of tuberculous tissue, it is not a question of an altered normal tissue. A *neoplastic* formation is in reality involved, for whose for-

[16] Mary, A. and A., and T. Pailly, *Le Médecin*, 1913
** Practically all precipitates formed *in vitro* result from the interaction of solutions that are colloidally protected. Because of this *plural* protection, such precipitates are, initially at least, in a very fine state of dispersion. See paper by J. Alexander on protection, Vol I of this series. *J. A.*
[17] Nolf, "La coagulation du sang," in "Traité du Sang," by Gilbert and Weinberg, Vol. I, p. 25, Paris, 1913.
[18] Widal and Sicard, Soc. Méd. des Hôpitaux de Paris, 1896.
[19] Jousset, Thèse de Paris, 1901.
[20] Howell, "The coagulation of blood," *Cleveland Med. J.*, 1910.
[21] Lumière, A., "Rôle des colloïdes chez les êtres vivants," Paris, 1921. (Also his paper in this volume. *J. A.*)
[22] Dominici et Ostrovsky, "Recherches, etc.," Paris, 1914.

mation no bacillus is essential, the agent being always of a toxic nature. Already in 1913, my brother and I proved experimentally that fragments of healthy tissue (liver, intestinal muscle of the guinea-pig) removed aseptically and grown according to the technique of Carrel, but *without renewal of serum*, show after a variable number of weeks, various histological changes of tuberculosis (appearance of figures showing mitosis). All this arises from the non-removal of functional by-products.[23]

Above all, remarkable results developed from our experiments with *micellular pseudo-tuberculosis*, whose production, course, and mechanism, we were the first to indicate (1913-1916). If a mixture of egg white and egg yolk is macerated under rigorously aseptic conditions with sterile bile, there are obtained, after about ten days, superficial whitish films which crawl up on the walls of the vessels, as though they were growing, and are composed of myriads of acid-fast micellular granules. Aseptic maceration of glycerinated liver gives a result of the same kind. These whitish pellicles, emulsi-
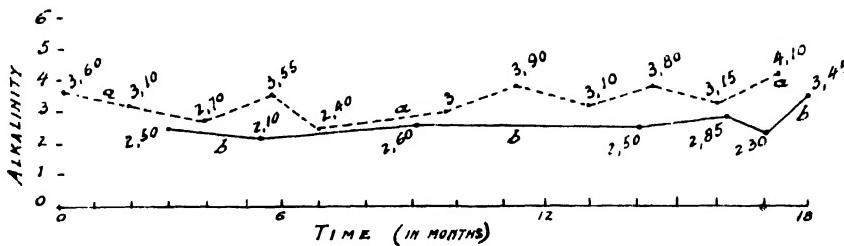
Fig. 1.

fied in physiological solution and injected into a guinea-pig, caused a progressive cachexia, and on autopsy pseudo-tubercules were found in the lungs and peritoneum. Microscopic examination of these pseudo-tubercules showed occluded capillary vessels, surrounded by a mass of cyanophile cells (embryonic elements of connective tissue).[24]

In 1917, in the histological laboratories of the University of Salamanca (Spain), Prof. Arturo Nuñez Garciá repeated and varied our experiments, and in reporting his observations[25] he does, in fact, insist on the divergences existing between micellular pseudo-tuberculosis and true pulmonary tuberculosis—divergences which are in reality less profound than Nuñez believed, as I have pointed out elsewhere.[26] But Nuñez did not stop there. Taking the micellular pellicles obtained with glycerinated liver, he triturated them in a mortar with powdered glass, so as physically to destroy the micells. The product of the trituration, filtered through a bougie and inoculated into the blood of animals, caused them to die of intoxication. Injected into connective tissue, it caused the growth of conjunctivo-embryonic vegetative nodules, veritable cumuli of cyanophile and polynuclear cells. Nuñez was thus confronted by true *experimental sarcomas*, arising out of attempts at cyto-culture.[27]

[23] Mary, Albert and Alexander, "Ensayo de Tisiologia," Barcelona, 1916.
[24] *Ibid.* See also A. Nuñez Garciá.
[25] Garciá, Arturo Nuñez, "Sobre la pretendida sintesis del bacilo de Koch," *Laboratorio*, 1917.
[26] Mary, A. and A., "Dictionnaire de Biologie physiciste," article on Tuberculose.
[27] Garciá, A. Nuñez, *La Semana Médica*, 1921.

This is not all. With rats cancer appears spontaneously on the breasts, and only occasionally may it be reinoculated in females of the same stock. But the number of successful transplantations, in general very meager, is increased by successive transplantations; the reinoculations then "take" in almost every case, and the tumors grow with unwonted rapidity and become transmissible to most breeds of rats. Nuñez selected one of these carcinomatous tumors; he enhanced its virulence by repeated transplantations, and when he had obtained a vigorous "cancerous matrix," he grafted it upon the breasts of rats previously inoculated with micells of glycerinated liver. He soon saw the occurrence of a transformation in the graft, and what was a carcinoma, evolved rapidly toward the sarcoma type. On the other hand, the cytocultures of human epithelioma, infected with the liquid resulting from the trituration of the micells, became transformed into cytocultures of sarcomatous nature by the rapid growth of the connective stroma and the atrophy of epithelial structures. What in the case of rats operated *in vivo*, occurred *in vitro* in the case of cytocultures of human epithelioma.

From these facts and various other considerations, Arturo Nuñez Garciá concluded that neoplasms are not caused by microbial agencies, but arise from a local *stimulus* or a trauma in organs or organisms more or less saturated with "mitosigenic venins," which are analogous to those formed by the aseptic disintegration of liver tissue in the presence of glycerin.[28]

As to the physical nature of the *mitosigenic action* of poisons, whatever be their nature or origin, we may state without fear of being mistaken, that there is involved a process of *pectization* of the cellular colloids. When submitted to destructive metabolism, whose end point is complete coagulation, certain organisms, *Saccharomyces, Hematococcus*, etc., tend toward elementary multiplication. In other words, the progressive destruction of the dispersed phase of histological colloids, is here, as in the artificial fecundation (parthogenesis) of Loeb, Delage, and Bataillon, a morphogenic creative agent of mitosic figures, and of unexpected proliferations.[29] In fact all experimenters, since Tichomiroff (1886), have used, for bringing about artificial parthenogenesis, reactions or physical processes of a coagulative nature: acids (Tichomiroff, J. Loeb, Delage), mercuric chlorid (Dewitz, W. Roux), hypertonic saline or saccharine solutions (Loeb, Delage, Bataillon), tannin (Delage), dehydrating trauma (Bataillon), heat (Delage, Bataillon), electric discharges alternately positive and negative (Delage), etc. Karyokinetic figures are furthermore observable outside of the protoplasmic cell, in colloidal substances, in the course of dehydration and pectization: precrystalline figures,[30] cell-like appearances on coagulation (L. Buscalioni), distorted crystals of cellular form or "protobic" nature (Herrera).

When Loeb and G. Bohn speak of the parthogenetic action of cytolytic or deflocculating agents (fatty acids, foreign sera, alcohol, ether, chloroform), they seem to lose sight of the fact that the nature of the reaction (dispersion or pectization) may depend on the nature of the colloid, and that an agent which is generally cytolytic, may nevertheless act as a coagulant towards certain protoplasmic colloids. Globulins, for example, are *coagulated* by chloroform, alcohol, ether, traces of alkalis, fatty acids—that is by cytolytic reagents (experiments of Porges and L. Bory on sero-precipitation; of M. H. Fischer

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[28] *Ibid.*, Mary, Albert, "Citogenesis patologica," *Rev. Heig. Tuberc.*, 1922.
[29] Mary, Albert, "Les Horizons du Physicisme," pp. 15-16.
[30] Mary, Albert and Alexander, "Sur la division karyocinétique des cristaux en formation," *Gaceta Médica Catalana*, 1919.

and M. O. Hooker on emulsoids).[31] Besides, the same reagent under certain conditions or simply in different percentage, exercises contrary influences. Mucins, though precipitated by small amounts of mineral acids, are redissolved by massive percentages of the same acids (Albert and Alexander Mary, 1913). Large percentages of alkali liquefy globulins, though small percentages coagulate (M. H. Fischer). Calcium salts in small amount accelerate the coagulation of blood, which they inhibit in larger percentage.[32] Viper venom, which coagulates blood in the proportion of 1 milligram per cc., is anti-coagulant in the proportion of 4 milligrams per cc.[33] Cytolysis and cytogenesis may thus be due qualitatively to the same physico-chemical influences, in whose action we must distinguish—as a function of quantity and perhaps also of time—an actual periodicity whose successive phases are opposed.[34]

I cannot close this section without pointing out that the resemblance between tuberculous or pseudo-tuberculous neoplasms, and cancerous neoplasms, is emphasized by the relative parallelism between tuberculous acidosis and cancerous acidosis. This parallelism is justified (1) biochemically, by the hypochloruria and the aceturia observed by Jaboulay; (2) physically, by the early diagnosis of cancer, based by Jules Regnault on the electrono-positive nature of cancerous energy: [35] we know that protein colloids carry negative electrons in alkaline aqueous solutions, and positive electrons in acid dispersions.

CASEIFICATION OF TUBERCULOUS TISSUE

When we see that tuberculous histogenesis follows changes in the degree of dispersion of the cellular colloids, we are not surprised that the final process of caseification has been attributed to a phenomenon of total coagulation.

Recently, S. P. Kramer [36] mixed intimately a 20 per cent solution of sodium silicate with an animal or vegetable oil, in order to obtain an emulsion. There is thus formed a combination between the free fatty acids of the oil with the soda of the silicate, whereby there is set free colloidal silicic acid which acts as a protective colloid. If a little lime water is then added, there is a precipitate formed, which resembles a coagulum of the casein type, because of the formation of calcium silicate at the expense of the free silicic acid. Kramer thinks that this illustrates what occurs in the organism when tuberculous caseification takes place. The patients deposit considerable silicates in their tissues, and these form with the aid of fatty substances, lipoids, and fatty acids, an emulsion which coagulates with the calcium compounds brought up by the blood. This point of view is in accord with the analysis of the caseous substance, in which my brother and I long ago mentioned the presence of silica, and in which Kohle found 1.60 grams, and Schweinitz found 5.70 grams per kilogram.

CONCLUSION

Tuberculosis should be studied mainly with reference to its relation to the physical chemistry of colloids. Although this point of view has as yet

[31] Fischer, M. H. and Hooker, M. O., "On the physical chemistry of emulsions," *Science*, 1916.
[32] Nolf, P., *loc. cit., supra*, p. 17.
[33] Noe, cited by P. Nolf, *ibid.*, p. 19.
[34] Mary, Albert, "Les Horizons du Physicisme," p. 17.
[35] Regnault, Jules, "Le diagnostic precoce du cancer par les réactions electronoque des réflexes visceraux," in *Compt rend du 28° Congrès de Chirurgie*, Paris, 1919.
[36] Kramer, S. P., "Pathologica," p 667, 1922.

merely been opened up, we see what light has been thrown on the genesis of the malady. Without doubt it will yield still more valuable information for the therapy of tuberculosis, which, from a prophylactic standpoint, should be of an *alkaline* and *detoxicating* nature.

Colloid Chemistry and Malignant Tumor

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That only one particular aspect of the relation between colloid chemistry and disease is treated here, demands explanation. As colloid chemistry is specially linked with life, and therefore with disease, all physiological processes and diseases should be explained with the aid of colloid and physical chemistry. This was well understood by J. Loeb, who devoted his latest researches to the study of the colloidal behavior of proteins, the most important carriers of life in animals.

We must apologize, therefore, when only one special disease is studied in this way. The reason is that malignancy reveals itself as a special *mode of growth*, and even in the growth process itself many colloid chemical principles apply.

Besides, attempts have already been made to solve the cancer problem by such methods, and previous work, namely by Clowes,[1] who has given many clues leading toward the understanding of malignancy, should be shortly reviewed here.

Cancer may be regarded as a special mode of reaction of a given cell to the *most diverse* stimuli. While in infectious disease specific organisms must find in the host appropriate conditions, varying within very narrow limits, the most diverse kinds of excitation may result in the development of a malignant tumor. *Thermal energy, radiant* energy (light, Röntgen rays), *chemical* substances, all may result by appropriate application, in producing tumors. Among the chemical substances mentioned, tar and tar products have shown themselves most active, so that the Japanese investigators, Yamagiwa and Ichikawa,[2] succeed in stimulating malignant growths in rabbits and mice by smearing them with crude tar. We are now able to study, by this method, the development of the tumor in its various stages, and to connect the various stages of the process with well known principles of colloid chemistry.

The primary alterations correspond to the process of growth, which is facilitated by the influence of the most various organic compounds; and they call to mind the experiments of Loeb [3] on parthenogenesis. All *capillary active* substances (ether, amyl alcohol, fatty acids) are able to excite a definite growth, though tar seems to have a more permanent influence. It is to be assumed, that, besides the property of capillary activity, definite chemical properties are necessary to the continuation of the stimulus. These properties are not yet known.

[1] Clowes, *Kolloid Z.*, September (1914); *J. phys. Chem.*, **20** (1916).
[2] Yamagiwa and Ichikawa, *Mitt. Med. Fakultat Univ. Tokio*, **15**, 1, 2, 4 (1916); 1917, 1920.
[3] Loeb, J., "Die chemische Entwicklungserwegung des tierisches Eies," 1906; "Studies on Parthenogenesis," Chicago, 1913.

Fig. 1a.

Fig. 1b.
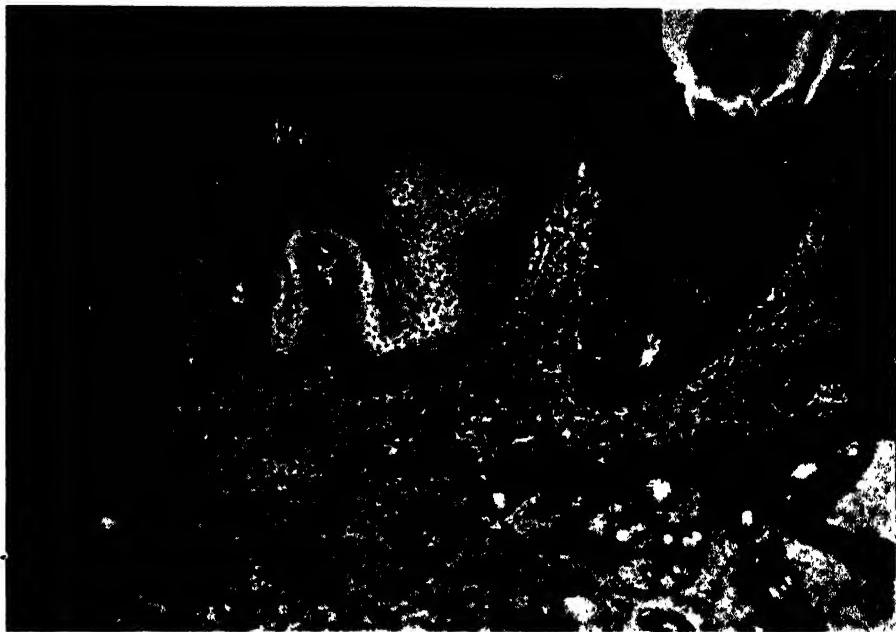
FIG. 1c.
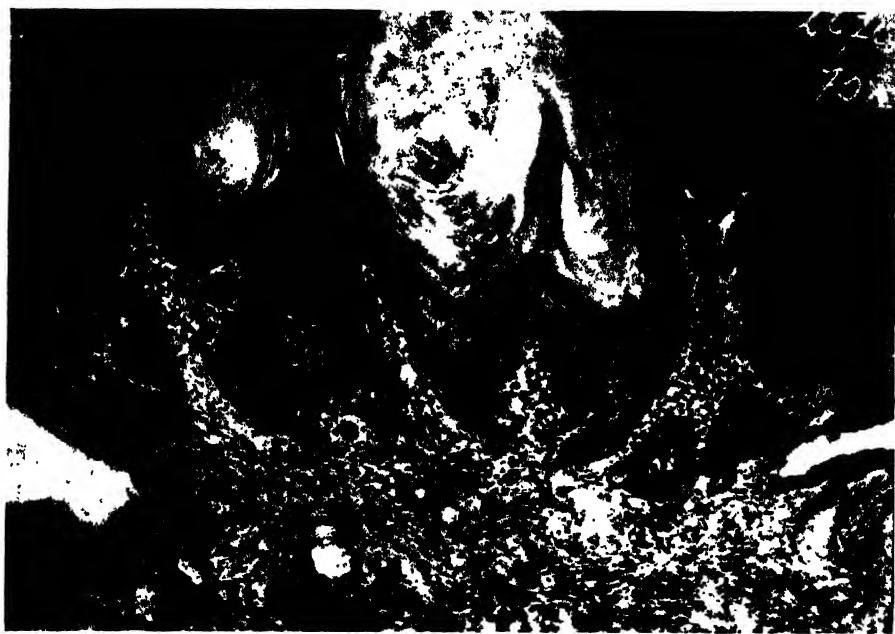
FIG. 1d.

It is obvious that after application of the chemical substance, cell division immediately begins, caused by decrease of surface tension of nuclear and cell periphera, whereby the globular form of cell and cell complex is altered, and the area covered by the mass of cells is enlarged. This process continues, when the stimulus persists. The accompanying photograph (Fig. 1) shows clearly, how first the cell mass is enlarged and, what is more interesting, how the surface is losing its globular form and transforming into the most fan-
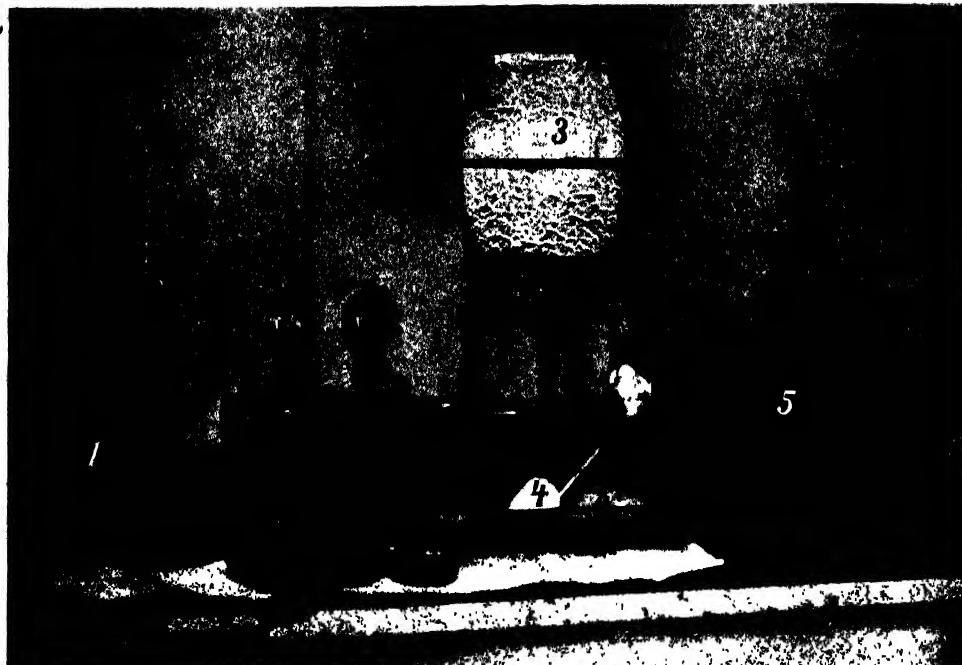
Fig. 2.

tastic and varied shapes, which themselves indicate the diminution of surface tension.

Another fact strikes our attention. Besides covering a larger surface, the cell contents appear clearer, which certainly is to be attributed to the fact *that the water content of each cell increases.* The photograph shows this peculiarity distinctly.

It is a well-known and most characteristic fact, that the water content of a tumor exceeds that of other tissues, and may exceed 90 per cent. The content, in general, varies with the rate of growth, so that rapidly growing tumors contain most water, the slowly growing, or retrogressing ones, less.

As Cramer[4] expresses this in anthropomorphic terms, the tumor cell is able to live and grow with less solid material than a normal cell.

This increased water volume is of considerable theoretical interest. We merely mention it now, in expectation of discussing it later on. It may, however, be said, that we are touching here the real basis of colloid-chemical

[4] Cramer, W., *Biochem. J.,* 12 (1918).

explanation of tumor behavior, as well as the controversy between Loeb-Donnan and the older colloid chemists.

The physical properties to be described now are, in all probability, consequent upon the two preceding properties.

It is well known that living tissue, though consisting of more than 75 per cent of water, offers a considerable resistance to the electric current. We must conclude that at the cell interfaces there exist impediments to the passage
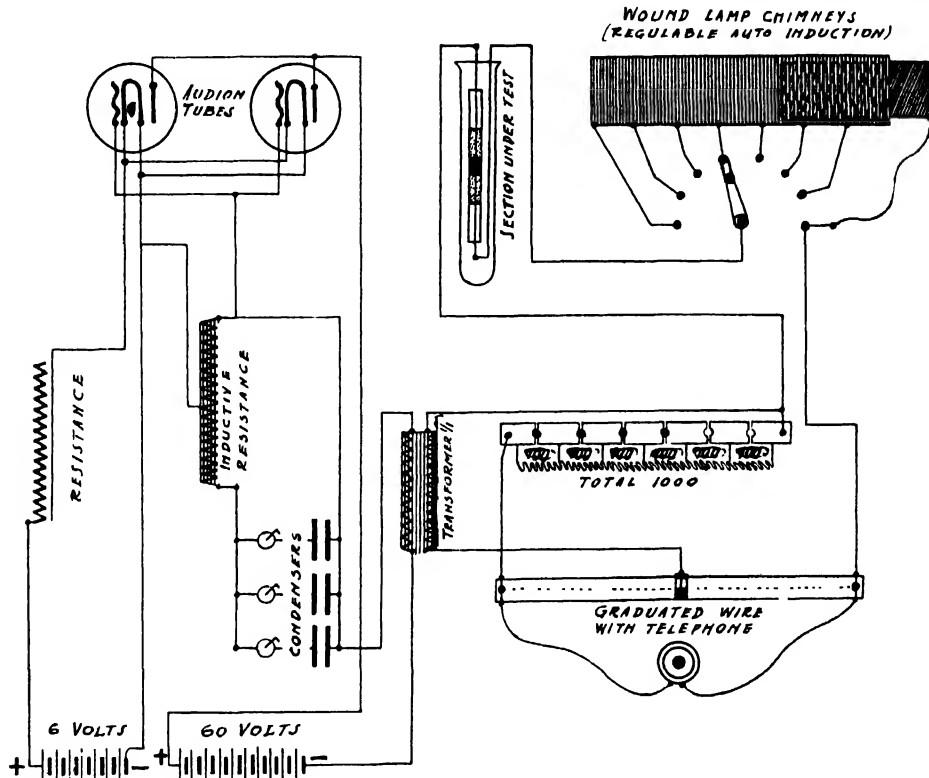
Fig. 3.

of the electric current. The resistance of a disc of tissue several millimeters in diameter, may amount to more than 1000 ohms. Now it is a very remarkable fact, that tumor tissue *has a far lower than normal resistance*. Fast growing mouse carcinoma or human tumor having a diameter of more than 0.5 cm. may show a resistance of only a few hundred ohms. Measurements of tissue resistance were made long ago, and elaborate investigations of this property were undertaken, e.g., by Osterhout and others, and the above described characteristic may be easily observed by ordinary methods.

In order to appreciate fully the theoretical and practical significance of these technical methods, it is, however, necessary to give in some detail the method to be followed.

It seems to me, that *one property* of living tissue, when traversed by an

electric current has not been given due consideration (except by Gilde-meister) ; [5] viz., that living tissue is highly *polarizable,* and that it is therefore necessary, in measuring tissue resistance, to control and to compensate the polarization either by a distinct amount of capacity, or by self-induction. When this precaution is omitted, the ordinary method of determining electrical resistance by a combination of a Wheatstone bridge with microphone and wire, fails to give a sufficiently sharp minimum and therefore gives inaccurate measurements.

When, therefore, a variable capacity or auto-inductance is included in the combination, the polarization is compensated, an exact minimum may be found, and at the same time the *degree* of polarization may be determined.

The annexed sketch gives an idea how the measurements can be performed (Figs. 2 and 3).

The alternating electric currents are generated by audion tubes; the fre-quency of alternation can be regulated by means of a couple of condensers. The apparatus needs no further explanation, as the method is one in com-mon use in physical chemistry. The variometer is constructed of lamp chim-neys wound with ordinary wire, of given diameter and length. The degree of auto-inductance can be easily calculated from the formula: $L = Qr^2ln$, where $Q =$ a constant, $r =$ diameter, $l =$ length, $n =$ number of windings, $L =$ auto-inductance; or better still empirically.

When the measurement is made in this way with normal tissues, it will be soon evident that there exists a relation between the actual resistance and the degree of polarization. This relation may be written as follows :

$$\frac{P.(\text{olarization})}{R.(\text{esistance})} = \frac{\text{Millihenrys}}{\text{Ohms}} = K = 0.015 \text{ (C)}.$$

This formula holds good for all normal tissues tested. In sharp contrast herewith, it is found in *all* tumors, not only in the cases above mentioned, that the resistance is reduced, but also that the polarization decreases to a much greater extent. Expressed in a formula, it may be written as follows :

$$\frac{P}{R} - \frac{MH}{O} = < 0.015 = \text{average } 1/3 \times 0.015$$

The data thus obtained is not only of theoretical interest, but will be found in the near future of some practical value, as this method permits us to deter-mine in fresh, unfixed tissue, the degree of malignancy. By a *modified form of electrodes,* we may, during an operation, distinguish tumor from healthy tissue (in case of doubt). The electrodes must be here, however, in needle form, and this brings with it serious difficulty, since with such a small surface, the electrodes themselves become easily polarized. This is prevented by cover-ing the silver electrode with a layer of silver chloride, and this layer again by a porous, salt-impregnated material, such as cotton wool or pipe-clay.

Table I gives some determinations in this way in normal and malignant tissues. In the meantime, these determinations have proved of practical value, not only in the hands of the writer, but also in some surgical clinics (e.g., in the Cleveland Surgical Clinic, Crile and Morse [6]) where every pathological examination includes and is controlled by the electrochemical estimation of resistance and capacity.

[5] Gildemeister, *Pflüger's Archiv,* 176 (1919)
[6] Crile (Morse). *J. Cancer Res.,* Sept. (1925).

TABLE I.

Sample	Thickness, mm.	Resistance	Polarization	P/R Coefficient	Remarks, Solution Employed
Skin of mice............{	3	530	3.5	0.0198	
	2	220	1.5	0.0204	
	4	558	3.5	0.0189	Ringer's solution
Skin of man	4	325	2	0.0183	
Skin of rat...............	2.5	630	3	0.0141	
Liver of rat.............{	5	1.380	8.7	0.0183	
	3	490	7	.....	
Kidney of mouse..........	5	610	3.5	0.0171	
Kidney of rat	5	1690	8	0.0189	
Muscle	3.5	675	3	0.0138	
Spleen	4	480	2.5	0.0156	Average: P/R = 0.0197
Carcinoma−mouse	5	505	0	0.	
Tar-ulcer (carcinoma)	1.3	255	1	0.0108	Ringer's solution
Cancer of human breast....	4	210	0.25	0.00357	
Glands with metastasis....	6	600	0.4	0.00201	
Cancer of the ovary.......	5	215	0.25	0.00330	
Cancer of the jaw	5	550	0.6	0.00327	
Papillomatous-carcinoma by tar application	4 5	690	2	0.00870	
Sarcoma of mouse........	6 5	660	1	0.00450	Average: P/R = 0.00401

Also, in more distant fields of research, namely, for the differentiation of the origin and treatment of imported meat, this determination has proved useful (Diemont).

Now, as we may expect from colloidal behavior, different ions exert a most important influence on form, surface tension, water content, resistance and polarization. This influence might have been predicted from the fact, that characteristic changes occur in the ash-content of fast and slow growing tumors. While in fast growing tumors the relation between potassium and sodium on one hand, and calcium on the other, is in favor of the alkali metals, the contrary is observed in slowly growing and retroceding tumors (Beebe,[7] Clowes, Frisbie,[8] Waterman,[9] and others). This holds good both for spontaneous human tumors and for experimental transplanted tumors in animals.

This fact, as yet not perfectly understood, bears a close relation to the general aspect of the problem.

Now the influence of the different ions may be easily demonstrated by the resistance-polarization method referred to. As was already shown by the sketch, the method allows us to examine the discs of tissue in different fluids. The original determinations were, of course, made in a solution of physiological composition, namely in Ringer's solution:

[7] Beebe, S. P., Am. J. Physiol., 11 (1904).
[8] Clowes and Frisbie, Am. J. Physiol., 14 (1905).
[9] Waterman, N., 5, 3, Archives Néerlandaises de Physiologie (1920-21).

NaCl .... 8.5 ⎫
KCl .... 0.02 ⎬ pH — 7.7
CaCl₂ .... 0.02 ⎪ per liter
NaHCO₃ .... 0.018 ⎭

The data given above, concerning the different values of P/R, all refer to experiments made in this fluid.

The most marked difference is observed when the relation between monovalent and bivalent cations is altered. Thus, when the tissue is immersed in an isoionic fluid having a predominant amount of calcium ions, definite phenomena occur.

While a normal tissue shows only slight variations in resistance and polarization (namely an increase of resistance, with corresponding degree of polarization, so that the P/R value shows a slight diminution), tumor-tissue reacts quite strongly in the opposite way, namely, by an enormous *increase* of the P/R value. The subjoined tables show the very marked difference in the mode of reaction of normal and tumor tissue. As may be seen, the P/R value may be increased by 100 per cent.

TABLE II.

Sample	Thickness in mm.	Resistance	Polarization	P/R Coefficient	Solutions, Remarks
Mouse sarcoma	{7	610	0.5	0.00246	Ringer
	{7	520	0.8	0.00450	CaCl₂ solution
Tar carcinoma	{7	870	0.8	0.0063	Ringer
	{7	450	1.5	0.0099	CaCl₂
Metastasis of breast cancer in lymph gland..........	{6	610	0.25	0.0012	Ringer
	{6	620	3	0.0144	CaCl₂
Cancer of the tongue.......	{5	198	0.3	0.00474	Ringer
	{5	204	0.5	0.0072	CaCl₂
Sarcoma fusicellulose	{5	750	2	0.0078	Ringer
	{5	800	4	0.0150	CaCl₂

Average *P/R* in Ringer's solution..................... 0.00468
   "   "   " CaCl₂   " 0.0111

These are fundamental facts, which must be explained. It may be further stated, that not only calcium, but *all* bivalent ions exert the same influence. *The effect is dependent on the electric charge of the ion.*[*]

The change in the P/R value is *immediate*, but the normal value of the P/R coefficient may be reestablished.

Now it is a curious fact that on immersing an original transplantable piece of tumor in the same isoionic calcium or other bivalent solution, this tumor tissue develops very slowly, if at all, in the grafted animal. *The bivalent ion has a growth-retarding influence*, and it is clear, that the phenomenon of P/R variation is closely linked with one of the most vital properties of the tissue.

Not only does tumor tissue behave differently from normal tissue in a lowered P/R coefficient, but it shows a markedly different response to the increase of calcium or other bivalent ion in the immersion fluid. In addition to the diminution of the resistance, this characteristic may also be of prac-

* It seems probable that there must be specific differences between ions of the same valency, even if present experimental methods do not always reveal them. See next paper by Dr. Waterman. *J. A.*

tical value, and has shown itself already of use in the diagnosis of human cancer.

Recurring to what was first said, we may ask, in dealing with the different modes of exciting malignancy, *at what stage* of the experimentation the phenomena described become manifest. With tar cancer we can give a definite answer, which so far is not the case in other experiments.

Tar gives rise, in a definitely fixed succession, first to hypertrophy of the epithelial cells, then to the formation of so-called papillomas, which sooner or later become carcinomas, at least (from an anatomical point of view) in so far as these cell complexes acquire now the property of invading the adjacent tissues, and of forming metastases.*

The electro-chemical method demonstrates conclusively that the characteristic tumor properties arise *suddenly*, in the second morphological stage, viz., in that of papilloma formation. The photographs illustrate the morphological sense of this. It is the stage where the surface tension of the tissue complex is decreasing, while at the same time the electrical resistance and polarization are diminishing, the P/R value declining, and the bivalent ion effect is appearing. The further development, the real malignant stage, merely shows these properties to a more marked degree.

Consequently, in our opinion, there is *no* real *biological* difference between a so-called benign or malignant formation, the only difference being a quantitative one.

To recapitulate, we have pointed out actual physical and chemical differences between normal and malignant tissues, viz., diminished surface tension, increased water content, reduced electrical resistance, diminished polarization, strong reaction upon the introduction of calcium and other bivalent ions, which restore the normal electrical resistance and polarization, and other inorganic chemical composition, in so far as the K/Ca equivalents are altered in a definite way.

These special qualities have been purposely enumerated, so that, starting from them, we may find a more or less satisfactory explanation, and see their significance from the standpoint of colloid chemistry.

It is evident, that in such a complicated entity as the living cell, it is nearly impossible to reach such exact conclusion as in the case of inanimate matter.

At first blush, it must seem striking that the degree of hydration (onkosis Schade)† must play an important rôle. Expressing ourselves in the terminology of classical colloid chemistry, we might say that the cell colloids of tumor-tissue are in a state of greater dispersion, or following Pauli's expression, the colloid particles are surrounded by larger water envelopes. Taking these expressions, as they are, as merely descriptive, we might imagine that where there is greater hydration and dispersion, the drop in electric resistance and in polarization are thereby explained, in addition to greater water content. When the different ions of the immersion fluids find larger channels along which they may pass, the lowered resistance is comprehensible; likewise the diminished polarization. In larger channels, neither the differences in velocity of anions and cations, nor their somewhat different rates of adsorption, come into evidence, so that no electrical contra-effects can occur, i.e., no polarization will arise. In like manner, the electric charges existent at the

surface of the particles, which tend to form double layers and increase the surface tension, disappear, causing the surface tension of the particles to diminish, and consequently there result changes in the form of the component particles, and secondarily of the tissue. Assuming, according to the Hof-meister series, the dehydrating effect of *calcium*, we understand its effect in increasing the resistance and polarization, when it increases in amount in the immersing fluids. This would be, I suspect, the customary "explanation" of the facts. This explanation is not a scientific treatment of the problem. As Jacques Loeb [10] remarked, an explanation of a phenomenon should be based on quantitative data and not merely on words.* Besides the "explanation" does not show us how to interpret the action of the tumor-producing sub-stances, or their effects. This does not mean that J. Loeb's own explanation of colloidal behavior of proteins by *Donnan equilibria* will lead us much further. Loeb tried, on the basis of the thermodynamic principles of Donnan (Donnan equilibrium) to explain the colloidal behavior of proteins, chiefly by studying gelatin. As colloidal behavior certainly includes reversible hydra-tion and ion effects, we must ask whether the new data are of more use to us in scientific understanding of our problem. Differences in water content, swelling, viscosity of proteins arise from particular equilibra, chiefly through the influence of changes in pH.

Changes in all these properties must lead to the same phenomena often mentioned above. Admitting a changed pH of the surrounding fluid in malig-nancy—a possibility to be discussed later—a different distribution of ions must occur in and outside of the tumor cell, accompanied by differences in osmotic pressure, swelling and viscosity.

As to the calcium effect, Loeb found and gave convincing evidence, that positive or negative ions act only in accord with their valency, not with their chemical properties, and that the Hofmeister series is an illusion,† due to the neglect of determining the pH of the solutions. As the pH of all animal and human fluids oscillates around the neutral point, or is on the slightly alkaline side, *all bivalent* cations must cause dehydration, and decrease in swelling and in viscosity.

The calcium effect is here explained as due only to dehydration, according to the ideas of the classical colloid-chemical school. While we must admit that these views are based on substantial experimental evidence, a most wel-come advance, it must nevertheless be said that they are not of much help toward the solution of our problem.

It is not clear, how the specific action of capillary active substances in initiating tumor is thus to be understood; nor is the action of other agencies causing tumor (heat and light action) explained. Furthermore, from the curves given by Loeb himself illustrating the bivalent action upon swelling of gelatin, it is apparent that at first the action is not very marked, and in the second place, no maximum exists in this action, as a regularly decreasing volume is noted in the experiments. All this leaves the different response of tumor and normal tissue unexplained.‡

that the dehydrating calcium effect *is not marked enough to explain the great physiological action.*

We have already referred above to the experimental work of Cramer,[4] who found that tumor cells after being immersed in Ca solutions, show diminished progagating properties after implantation. The same investigator stated, in fact, that tumor cells, after being immersed in Ca solutions, lost *more* water than in the corresponding Na and K solutions. But the differences are very small, as shown by Table III.

TABLE III.   *Carcinoma Cells of Mouse.   Water Content in Isotonic Solution.*

Na$^+$	Ca^{++}	Na$^+$ after Ca^{++}
85.4 per cent	83.8 per cent	86.8 per cent
85.1 per cent	84.0 per cent	85.2 per cent

Osterhout, in his well-known experiments on *Laminaria*, also reaches the conclusion (page 180) [11] that the Ca″ effect on swelling and viscosity alone can not account for the considerable effect on permeability and electrical resistance.

Our conclusion must be then, that the special physio-chemical properties of tumor tissue are yet unexplained by the older colloid chemical explanations, as well as by the new experimental work of Loeb and his collaborators, which is founded upon the Donnan equations.

It is, I think, not at all astonishing that the problem cannot be solved in a comparatively simple manner. For the cell is not a very simple biphasic system, of solvating and solvated material, but a very complex multiphasic, heterogeneous one. It is probable that the cell content is best comparable with an *emulsion.*[*] and it seems possible, making use of the work of Bancroft [12] and Clowes [1] to reach a better insight into the different properties of normal and malignant cells.

According to Bancroft, it is essential for the stability of an emulsion, e.g., of oil in water, that the emulsified particles be as small as possible; and second, that they be prevented from confluence by a third substance, enveloping the particles and segregating them, chiefly by giving them an electric charge of the same sign.

We may conceive of the cell as an heterogeneous emulsion, where the hydrophile substances, proteins, etc., include fatty substances of definite but very complicated composition, comparable to a cream.

It is easy to perform model experiments with the oil-water system outlined above. And it is with this model that Clowes reached very interesting conclusions, which throw more light upon the resistance phenomena in tumor cells, than the other explanations cited above.

When an oil-water emulsion, e.g., of olive-oil and water, is made by adding alkali (caustic soda) to the fluid, we have a creamy emulsion, wherein the oil

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[11] Osterhout, "A monograph on experimental biology: Injury, recovery and death in relation to conductivity and permeability," Lippincott, 1922.
* In his book on "The Influence of Colloids upon Crystalline Form and Cohesion" (1879), W. M. Ord refers to the work of Ascherson (*Müller's Archiv*, for 1840, p. 44), which shows that when oil and albumen are shaken together, an emulsion is formed which is permanent, because every globule of oil is instantly invested by a pellicle" (page 11). Ord repeated the experiments, and made interesting observations on the Brownian motion of the emulsion particles, their activation by alkalis, and their inhibition by acids. Ord attributed the motion to chemico-molecular change of the colloid (albumen), but says that Prof. Jevons considered electrical relations to be responsible. *J. A.*
[12] Bancroft, *J. phys. Chem.*, **20** (1916).

particles, covered by a layer of sodium soaps, are dispersed in the water. This emulsion easily conducts the electric current, because the ions will move easily in the spaces between the oil droplets. The more alkali added, the more the resistance will be reduced.

A wholly different condition will arise now, when *bivalent cations* are added to this creamy emulsion. At first the resistance will be lowered still further, because the solution contains more ions; but, suddenly, when a certain proportion between Na⁺ and bivalent cation (Ca⁺⁺) is reached, the conditions are changed. At a certain point, soaps of bivalent cations are formed, instead of those of Na (or K), and then bivalent soaps having a greater affinity for the oil phase, their surface tension at that side is diminished so that a very interesting transformation of the emulsion takes place—the oil surrounds the hydrophile portion of the emulsion. This process may be examined under the microscope to watch the beautiful play of changing forms; it is possible, too, to see the process with the naked eye, by staining the water and oil phase with different colors.

The result will be understood easily. Instead of a creamy emulsion, we have now an emulsion comparable to butter, where the aqueous phase is surrounded by the fat.

The consequences will be that the electric current will meet now with a *strong resistance*, as the ions cannot at all, or only with great difficulty, enter the oil, and during the phases of transition the ions must wander through elongated pore-like channels of small diameter. That in this condition resistance and polarization will be very high, is obvious.

The accompanying curves (Figs. 4 and 5) demonstrate the process very clearly, though, of course, these experiments and models are very crude. The cell is no emulsion of water and oil, but nevertheless this reversal, which we may call henceforth in honor of its first investigator, the *Clowes-effect*, is a very important biological phenomenon, explaining * many different life phenomena.

First, it is noteworthy, that the point at which the sudden change takes place, is fixed, and is the point, where, as in all balanced (Loeb) fluids, a definite relation exists between monovalent and bivalent cations. This fact shows itself at a very definite molecular ratio, and it is possible to fix the physiological balancing of salt solutions by determining the point C of the curves. This is significant of the great physiological importance of the model. A very curious fact is that the Mg ion occupies in this model the same intermediate position that it does in all other biological phenomena. It permits also an understanding of the phenomena of permeability which have aroused so much interest in the last few decades of medical investigation. It will be apparent, from the crude model, how under the influence of different ions, different substances may penetrate to a very different degree. Not only is the chemical composition of a substance of the highest importance in connection with the phase of the model, but we may also imagine the *structural* forms to be of interest, when the different forms of pores are considered. The explanation of Clowes was therefore accepted by Hamburger, in his work, as explaining the wholly different behavior of α, β, and γ glucose in the course of elimination by the kidney.

It would lead us too far, to go further into this matter; we must return to the tumor problem.

* This is too broad a statement. *J. A.*

Evidently the tumor cell is defined by saying that it is more hydrophile, in a more cream-like state of emulsion, than the normal cell; hence the lowered resistance and polarization.   Hence also the enormous sudden response to the addition of bivalent ions, with the rise in resistance and polarization.

While in the normal cell there seems to exist an equilibrium between monovalent and bivalent ions, this relation seems disturbed in the tumor cell, there being a preponderance of monovalent ions. This view harmonizes with the before-mentioned results of chemical analysis of tissue ash, and also with the very probable increase in the alkalinity of tumor tissue compared with normal tissue. This last fact is not conclusively proved, and we must return to this question in discussing serological features in malignancy.

That the response to increase of bivalent ions in the immersing fluid may be different in normal and malignant tissue, and that in normal tissue further Ca increase may result in a reduction of the P/R coefficient, will be perhaps
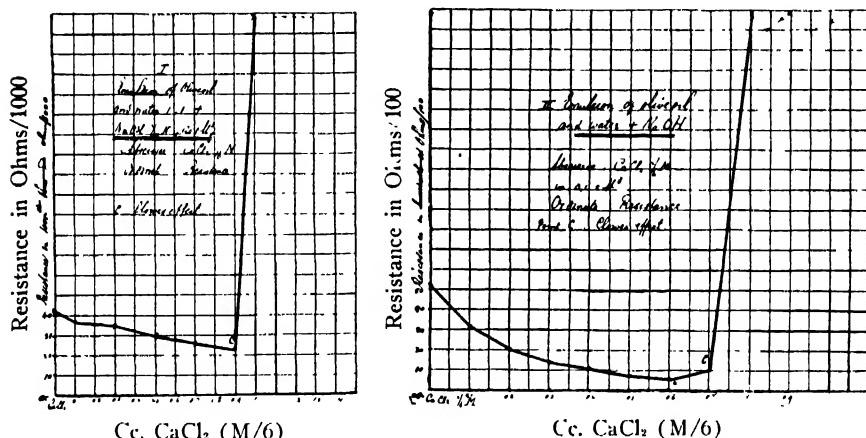
FIGS. 4 AND 5.—Changes in Resistance in 120 cc. of 50% Emulsion of Olive Oil in 0.1N NaOH, following progressive addition of M/6 CaCl₂. C = Clowes Effect.

understood, when we admit with Neuschloss,[13] that lipoids show a maximum of lowering of surface tension *with a definite ratio between monovalent and bivalent ions*, and that on changing quantitative conditions, the reverse takes place.

Though these model experiments and explanations may seem crude, and they surely are so, it cannot be denied that they give a better explanation of the facts observed in malignant tissue, than do other physico-chemical notions.

Undoubtedly many changes must be made in these views. One positive incongruity is the idea that the interfacial substance consists of soaps. Its chemical composition is far more complicated, and between the component parts antagonisms already exist.*

More than 10 years ago the French investigators, Mayer and Schaeffer,[14]

¹³ Neuschloss. *Pflüger's Archiv*, **180** (1921)
* This is naturally so in the operation of cumulative colloidal protection   See Vol. 1, this series, J. A.
¹⁴ Mayer and Schaeffer, *J physiol. Pathol. Gen*, **15**, 16 (1914-1915).

showed that for every normal tissue there exists a fixed relation between the cholesterol and the phosphatid content, both cell constituents of antagonistic physiological function, both belonging to the ubiquitous lipin group. As the function of both substances is completely antagonistic, their unequal distribution individualizes the cell.

An important distinction between both substances lies in their behavior towards water. While the phospholipins are very hydrophilic, the cholesterols are absolutely immiscible with water. And a curious fact, stated by these investigators: in carefully conducted determinations, *the water content of a cell depends on the quantitative relation between phosphatids and cholesterol.*

It seems very probable, that in the tumor cell there exist quantitative differences in the distribution of the lipins and that, e.g., preponderance of phospholipins occasions increased water content. Indeed, the preponderance of soaps and phosphatids in the tumor cell is claimed by many, though as yet we are without positive confirmatory evidence.

It is obvious how Clowes' views must be amplified. It becomes also comprehensible how all influences (e.g., tar), which act upon the interfacial fatty- or lipin-layer, must have a considerable effect upon all the physical properties of the cell, thus far broadly treated. It is clear also how by continuous application of tar, physical conditions may arise, corresponding to those regarded as being essential for tumor cells.

A few words must be said on the general reactions of the whole organism in malignancy. So far, only the tumor cell has been considered; but disease involves abnormal reactions to known deviations, as well as the existence of these deviations. In cancer the influence of the "milieu" should certainly not be disregarded.

In our preceding studies and explanations, we have seen, that for the development of the tumor cell it must be of fundamental interest, whether the milieu in which it is immersed is acid or alkaline: whether the milieu should contain more or less calcium ions, etc.

The reaction of the fluids dominating animal life is remarkably constant. This does not mean that there may not exist trivial individual and temporary deviations in healthy and pathological conditions.

Now all investigators, who have studied blood reaction in human cancer, have reached the conclusion (Morazewski,[15] Moore,[16] Menten,[17] Burrows,[18] Waterman [19]) that the reaction is slightly changed toward the alkaline side. This fact must be of considerable importance in the further development of the malignant cell. We have seen from the experiments of Clowes, how increased Na^+ content tends to increase the hydrophile state, the creamy condition of the emulsion; further we must consider how the Ca^{++} content of the surrounding fluid is regulated by its alkalinity.

What we do not know at present is, whether the increased alkali content is primary or secondary. In the first case there must exist in the organism a deficiency in the reaction-regulating factors; in the second it would seem that tumor material is secreted into the blood stream, and that this tumor material has a more alkaline reaction than the blood. In both cases, however, the once established "alkalosis" must dominate further tumor development.

[15] Morazewski, *Virchow's Archiv* (1894).
[16] Moore, *Biochem. J.*, 1 (1906).
[17] Menten, *J. Cancer Res.*, 4 (1917).
[18] Burrows, *J. Cancer Res.*, 6 (1922).
[19] Waterman, *Biochem. Z.*, 133 (1922).

Another physical alteration in the blood composition, undoubtedly secondary to tumor development, deserves attention. It has been demonstrated that the surface tension of the serum of the tumor carrier has decreased.

This change is most clearly demonstrated, if to the serum is added a substance (antigen), which likewise lowers surface tension. When we add to serum, e.g., linoleic or ricinoleic acid, in equivalent amounts, and we heat the mixture for 1 hour at 50°, we see an important difference between a normal and a cancerous serum. The latter is far less able to combine with the added fatty substance, so that the latter remains free, and gives rise to a far greater diminution of the surface tension than is the case with normal serum.

This different behavior, pointed out by Ascoli,[20] is known as *meiostagmin reaction*, and has proven useful as a serological diagnostic method for cancer.

The above fundamental fact harmonizes quite well with the views expressed in the discussion of the Clowes phenomenon. As will be recalled, it was there pointed out that this scheme should be modified, and that the interfacial layer or third phase should not be regarded as a simple layer of soaps, but as a far more complicated structure, composed of many substances to some extent functionally antagonistic.

It was there stated, in referring to the work of Mayer and Schaeffer, that the character of a cell is partly determined by the proportion of these antagonistic substances, viz., chiefly phosphatides and stearines, and some experimental evidence was adduced to show that there exist in the tumor cell a rather different proportion, with preponderance of the (hydrophilic) phosphatides.

When parts of tumor cells are dissolved and excreted into the blood stream, it becomes evident that a diminution of surface tension will occur, a diminution which will become still more obvious, if definite tension-reducing substances are added. In this manner serological examination confirms the ideas already expressed when studying the changes in the tumor cell, apart from the importance this test has in tumor diagnosis.

Summary

It is pointed out, that malignancy is, in many respects, if not in all, a question of peculiar colloid chemical behavior, and may be explained according to the principles and methods used in this branch of science.

Characteristic aspects of the tumor cell are: increased water content, decreased surface tension, reduced resistance to the electric current, with reduced polarizability, and large and sudden reaction to the addition of bivalent ions in the surrounding fluids.

The properties enumerated account to a large extent for the so-called malignant behavior, and may be used in diagnosis. The determination of the $\frac{\text{Polarization}}{\text{Resistance}}$ constant by a special apparatus will prove itself especially useful in further investigations of malignancy.

On the other hand, it must be admitted, that existing theories do not account fully for all observed phenomena; neither do they give a sufficient clue to the understanding of the origin of the changes noted. This concerns the older ideas of colloid chemistry as well as the newer ones advanced and advocated by J. Loeb.

This is not at all surprising, for the phenomena in question are far

[20] Ascoli, M., *Münch. med. Wochenschr.* (1911-1912).

more complicated than those studied by the different authors in experimental models.

Attention is drawn to another form of explanation of the occurrences observed, namely, to the experiments of Clowes, based on the emulsion theory of Bancroft. The work of Clowes is founded upon the assumption of presence of a third strongly surface-active phase, which separates the two others and which can be affected by different actions. The relation of the two other phases is dependent on the condition of the third. The electric phenomena can be explained on this assumption, as well as the influence of bivalent ions. However, the Clowes scheme must be amplified, since the third phase is certainly not homogeneous, but heterogeneous. The theory of antagonism between different lipin substances must be applied to this layer.

Malignancy should not be regarded merely as the presence of tumor cells in the organism; the reactions of the organism with regard to these cells are also of fundamental importance.

Among such reactions, the degree of alkalinity of the blood may be studied provisionally; further the reaction with respect to the addition of capillary active substances (meiostagmin reaction) is of theoretical and diagnostical interest.

Cancer, Selective Fixation, Curiegraphy and Organoradiumgraphy

By Dr. A. Kotzareff

Laureat of the Paris Academy of Medicine.*

General Prevalence of Cancers

For many years we have studied the cancer problem in its clinical and experimental aspects, that is, we have sought to find the ultimate cause of cancers, to make an early diagnosis, when the malady is still in the pre-cancerous stage, and finally to develop a local and general treatment of the cancerous organism.

Cancer should be considered under general biology, because it is a cellular malady. In the case of the human being, as with the animal, the cell, before becoming cancerous, passes through several stages. Thus, for instance, an adult cell, before differentiating itself, will return to an embryonic stage, then, if the division is normal, typical, it divides without invading the adjoining tissues and without metastases, the tumor formed being a benign tumor, which once removed will not recur. If, on the other hand, the division is asymmetrical, atypical, with invasion and destruction of the neighboring tissues and recurring after ablation, the growth is called a malignant tumor or cancer.

Let us investigate what agents are capable of perverting the normal cellular division. They may be *physical* (X-rays, radium, light, etc.), *mechanical* (grattage, friction, puncture, etc.), *chemical* (hypertonic liquid, lytic substances, paraffin, tar, arsenic, etc.), and *biological* (microbes, parasites, spermatozoa).

All these agents act on the most exposed part of the cell, that is to say, on its wall. The physico-chemical equilibrium of the colloidal micells which make up the cellular membrane is modified, whether the agent be chemical or biological. The micellary lack of equilibrium will affect all functions of the membrane, particularly its permeability, and secondarily all cellular metabolism, and specially on the nuclear metabolism which is always sensitive to external influences. This increase of cellular permeability is accompanied by increase in surface tension, by variation of the pH, a release of anions, and a variation of the global potential of the cell in the process of division.

The division starts with a strangulation of the cell, which would be due to an increase of the surface tension localized at the two poles of the cell (Lillie).

If this localization is not balanced or is disturbed, there will be asymmetry, several short asters of atypical form, which establish a morphologic primordial characterization of the cancerous cell. To this it may be objected

that typical cell division is not like cancerous cell division. Claude Bernard has said: "There is no such thing as a physiology and a pathology, but only a single physiology, pathology being a question of degree." A disturbance in the mechanism of typical cell division may, therefore, account for the atypic division.

Thus cancers are due to a disturbance of the *photo-chemical* (*electro-radio-chemical*) equilibrium *of the normal cellular metabolism leading to an atypical division.* This parthenogenetic cause, acting similarly to the one taking place in the egg (J. Loeb, Delage, Bataillon, G. Bohn, *et als.*) will bring about cellular death, if the conditions and the equilibrium permit; but considerable variations in pH, resulting from ionization and the chemical relations between the medium and the cell, partly neutralize this action and maintain the life of the cell. An equilibrium in these tendencies leads to proliferation and the evolution of cancer.

However, how are we to explain the invasion of the neighboring tissues and the destruction of organs by cancerous cells, that is, how does a cell, confined in a basal membrane, as in the case of a cancer of the skin, come to clear, or break, this membrane and penetrate into the derma? The results of Gurwitsch indicate that a cellular group in the process of division emanates a radiation called the "mytogenetic radiation". This radiation may act over a distance of at least 1 cm. The researches of Berthelot indicate that ultraviolet radiations and, indeed, all radiations, may produce the synthesis and the decomposition of inorganic or organic substances, without the intervention of ferments. Thus digestion or lysis may be effected under the influence of radiation. If we admit the existence of mytogenetic waves in the case of atypical division—a radiation far more intense than in the case of typical division—we can explain the lysis of the confining membranes. This lysis, however, may be of a chemical, electrical, or luminous origin; hence we may have chemolysis, electrolysis, photolysis, or, to use a general term, radiolysis. A chemical reaction always involves absorption or emission of energy, that is, an absorption or an emission of vibration. Now the various radiations, known under the name of X-rays, γ-rays, ultraviolet rays, luminous rays, Hertzian waves, are vibrations of the same nature; they only differ in vibratory frequency.

However, in the cell, especially in its membrane, there exists a radioactive element—potassium (K). J. J. Thomson has demonstrated that the K emits penetrating β-rays but few γ-rays. We know, furthermore, that in cancer the K increases in quantity. Furthermore Zwardemaker has demonstrated that the K is indispensable for life. Finally, according to J. Perrin, "protoplasm functions poorly if it is not irradiated with a fixed intensity by the rays emitted by radioactive atoms—(potassium or its equivalent). There is a lack of equilibrium somewhere, and I ask myself whether the search for the origin of cancer should not be made along this line."

In a general way, under the influence of radiation, metals emit electrons; and as a result of atypical cellular division, its mytogenetic radiation, diminution of the permeability of the cellular membrane, the presence of a large amount of K and other unknown factors, may explain the invasion and the lysis of the adjoining tissues by cancerous cells.

We think that this same process explains the lysis of vessel walls by cancerous cells, neoplastic thrombosis, cancerous embolism and, finally, the metastases.

So far we have discussed the physico-chemical modifications in the can-cerous cell; yet one cannot conceive the life of a cell without the existence of its milieu. The milieu supplies to the living cell its component substances and available energy. There is a continuous exchange between the living cell and its milieu. Consequently if, in the course of cancerization, the one suffers physico-chemical modification, the other cannot escape.

Have we proofs of the physico-chemical modifications of the milieu (blood, lymph, interstitial liquid)? Most assuredly. These modifications are both chemical and physico-chemical.

Chemical Modifications. Nitrogenous substances (globulins, amino acids) increase in quantity proportionately to the growth of the neoplasm. The blood shows a larger proportion of lecithin, with an increase of the lipocho-lesterin ratio, a rise of the non-protein nitrogen, and of glycoproteins which may increase five times. The increase in amino acids is demonstrated by the colorimetric tyrosinase-paracresol method (Chodat-Kotzareff). There is an increase in potassium (Waterman-Loeper), elevation of the fatty acid level. The pH of the plasma is lowered. Reduction of the basal metabolism, evidence of hypothyroidism (Sendrail).

Physico-Chemical Modifications. Cancerous serum, as compared to normal serum, shows granules which are larger, less numerous and less mobile, and flocculates can distinctly be observed in the ultramicroscope. Cancerous serum is far less stable than normal, its lability being largely due to modification of the electric charges of the cancerous proteins.

The flocculates in cancerous serum disappear as soon as the serum is subjected to radium emanations (Kotzareff-Fischer).

In short, it seems evident, as we have maintained for some years past, that cancer destroys the electro-colloidal equilibrium of the body fluids.

What has heretofore been said applies to human and experimental cancers. We have observed chemical and physico-chemical modifications even in the pre-cancerous stage (chronic diseases, papilloma, fibroma, hyperplasia), that is to say, in the stage of "biological malignity" (Bang). However, none of these modifications is specific so as to enable us to make a positive diagnosis of cancer.

To summarize. Cancer is a local, cellular malady, developed on a former lesion (pre-cancerous condition) which was not treated or treated badly. Consequently cancer is a local but secondary malady. The factors which determine it may be of different kinds—physical, chemical, biological,[*] but their action or their mechanism is alike, physico-chemical or photo-electrical (radio-chemical).

The diagnosis and the treatment of cancers must be of the same kind, physico-chemical. As there are local and general modifications of the cancerous organism, a local and a general treatment must be employed. This is why for some years past we have centered on this point, namely, local application of radium (or local injections of radio-active sub-stances, operation, X-rays) and intravenous injections of radio-active sub-stances.

From these intravenous injections of radio-active substances the idea of *selective fixation, curiegraphy,* and *organoradiumgraphy* was born. This method of introducing radio-active substances into the circulatory system serves both for the diagnosis and treatment of cancers.

[*] This refers to both human and experimental cancer.

By selective fixation we understand the fact that a substance introduced into the organism and circulatory stream by different ways (whatever the initial way of introduction, intravenous injection, local injection, ingestion, or inhalations) will be preferentially adsorbed by a system, an apparatus, an organ, or a cellular group. Selective fixation is to be taken relatively. It is the basis of the biochemistry and of all therapy which aims to affect certain cells of the organism to the exclusion of others, or to inhibit the action of certain parasites.

Experimental studies on radio-active substances have led to special consideration of the selective fixation of these substances by embryonic cells in general, and by neoplasms in particular.

EMANATION OF RADIUM

In 1904, Ch. Bouchard, Pierre Curie and V. Balthazar studied _the physiological action of radium emanation_ on mice and guinea-pigs. In a first series of experiments the emanation was inhaled. The _dead animals_ 4 to 9 hours later were placed on a photographic plate enclosed in black paper. The body of a guinea-pig gave an image showing the hair very clearly. Three hours after death, and by the same process, the radio-activity of the various tissues was examined:

"All are radio-active, but in _variable degrees_. The radio-activity attains its maximum with the hair. The hairless skin and the eyes are least radio-active. The kidney, heart, liver, spleen, and brain have the same intensity; curiously enough, it is much greater in the suprarenal capsules and in the lungs."

These pioneers already dissociate the causes of this radiographic action.

"This radiographic action depends on two causes: (1) The induced radio-activity of the tissues (induced radioactivity is the radioactivity produced by the products of decomposition of the emanation which deposit on the walls in contact with the emanation liberated); (2) the presence of emanation in the humors; _it would be interesting to separate them_."

In the same year London studied the physiological and pathological value of the emanation of radium and of the radioactivity induced by inhalation, on frogs and mice, and the work of Bouchard, Curie and Balthazar was confirmed. These frogs die between the 12th and 14th day in an atmosphere of emanation; the water in which they live becomes radio-active.

These dead frogs were exposed on radiographic plates (this, according to London himself, results from an induced radioactivity). In fact, when he cuts off a disc of the skin on the back and then exposes the frog, he obtains an image of the outlines of the animal in which the region of the removed skin is missing.

Hence everything not skin (i.e., muscles and bones), that is, everything not in direct contact with the deposit of the emanation, is not radio-active and does not affect the photographic plate.

In 1906 Bouchard and Balthazar once more took up the matter.

They submit guinea-pigs and rabbits to the action of radium emanation disengaged by radiferous barium sulfate placed in a collodion sac and aseptically introduced into the peritoneal cavity.

They determined the quantity of emanation contained in each organ by

measuring the conductivity of the gases extracted from them by a mercury pump. The conductivity was measured by the P. Curie *quartz-piezo-electric method.*

They found for

3.60 grm. of lungs	59.95
4.70 grm. of kidneys	54.74
0.68 grm. of suprarenal capsules	2.77
0.75 grm. of spleen	12.00
3.50 grm. of skin	8.50
18.50 grm. of liver	6.15

but, reduced to unit weight per organ, it will be seen that 1 grm. of suprarenal capsule contains 4.7 times more emanation than 1 grm. of spleen and the descending scale of the organs containing emanation is the following: suprarenal capsules, lungs, liver, skin, spleen, kidneys.

By subcutaneous injection of emanation Bouchard and Balthazar observed that the emanation localizes itself after the second hour especially on the suprarenal capsule, which, in the 4th hour contains as much as the rest of the body. Six hours after injection, the body no longer contains any emanation.

The authors, therefore, believe there is a selective localization of emanation in different organs.

Lazarus caused emanation to be ingested by mouth and found it shortly afterwards in the saliva, sweat, placenta, body of the foetus and also in the mother's milk.

RADIUM SALTS

Dominici, Simon Laborde and Albert Laborde have studied the action of radium salts injected into the organism (rabbits). The results differed entirely from those just mentioned. The term of life of the injected radio-active substance, its mode of absorption and elimination cannot be compared in the two cases. According to Bouchard, Curie and Balthazar radium emanation disappears in the course of six hours. Dominici, S. Laborde and A. Laborde still find 50 per cent of the injected substance *25 days later*; and 10 to 5 per cent 90 days afterwards.

Here it was again a case of *insoluble* salts being injected into a vein. The same *insoluble* salts of *radium sulfate* injected into the muscles, remain for the most part at the point of injection to the detriment of the body and of the organs, and are less active than if the injection had been made into the veins. It is eliminated very slowly; 131 days afterwards 50 per cent of the quantity injected may still remain.

The localisation of *salts insoluble intravenously* follows a chance distribution, both vascular and through the different viscera.

With soluble salts, radium bromide, we find that elimination is slightly more rapid; even when injected into the muscles they diffuse into all the organs. The kidney and the digestive tract fix it partially, which seems to indicate that the product is eliminated in these organs. But it *fixes itself* particularly and in appreciable quantity in the skeleton and the bone marrow. Dominici, S. and A. Laborde have verified these results in other papers published by the Biological Society of Paris.

In these results a distinction must be made between various localisations.

When iron is found in certain parts of the intestines and bismuth or Hg on certain mucosa, we consider that these substances are being excreted, that there is an eliminative fixation as we find in the liver and kidneys of animals; we deal here with avenues of elimination.

Everything else is fixation by the skeleton or the suprarenal capsules; this is selective fixation.

These facts have been verified with various radio-active substances: active radium deposits (Bagg), mesothorium (Bickel), radiothorium, actinium (Lazarus), thorium X (Plesch, Falta).

Research work with thorium X has shown that this radio-active substance is particularly efficacious, and thanks to its short term of life it is innocuous in appropriate doses.

Actinium. Lazarus has extended our knowledge of this material by the study of intravenous injections into the tails of rats. After death he finds the "autoradiograms" of liver and skeleton; the image appears clearer with the emulsion of actinium than with the filtrate.

Later the action of radium emanation was studied on *living beings.*

We have given particular attention to this problem which is far from being exhausted (1921). We will here state the results found.

(1) Study of the radium emanation, then

(2) A mixture of emanation and of traces of polonium not over 100 electrostatic units, which we have called "poloniated" colloidal radium (radium colloidale "poloniée").

For the next two years we again employed the emanation of pure radium.

After us, Lacassagne, Lattes and Lavedan have studied polonium, and Ferroux and Lacassagne have experimented on the radium emanation

As regards the injection of polonium, if the animals did not die, these scientists killed them and then exposed their organs, finding a rather uneven fixation according to the organs considered. They found that, with certain doses, the radio-activity persisted for three months after a single injection. The main fixation is in the reticulo-endothelial and "blood-forming" organs, the kidney, lung, and suprarenal capsules.

Ferroux and Lacassagne have experimented on normal rabbits and grafted rats, and they confirm the selective fixation of emanation by the neoplastic cells. However, the animals were killed immediately after the injection; but, as we shall see, before fixing itself, the emanation is uniformly distributed.

Rats with grafted tumors were injected *locally* or into the peritoneal cavity and immediately afterwards placed on the photographic plate. It seems obvious that under such conditions the place of injection should show the strongest impression.

Finally no grafts have normal vascularization and offer numerous areas for necrosis; hence we do not think that these authors have demonstrated the non-existence of selective fixation.

The same authors have injected radium emanation into the jugular of cancerous (grafted) rats, and they say: "the trace left by the tumor (Curiegraphy) both with the entire animal and with isolated organs, was very much fainter." Here two points must be remembered: (1) In the case of the living animal with a tumor, the tumor alone has affected the plate; the intensity of the impression matters little, since the other organs do not affect the plate while the animal is alive. Hence there is selective fixation by the tu-

mor. (2) The same organs which, exposed after the death of the animal, affect the photographic plate more strongly than the tumor, would not affect the plate (the animals being still alive) in spite of their fixation being stronger than that of the tumor.

Solutions Employed as Vehicles of Emanation

As the emanation is an inert gas, it will follow the solubility law of such gases. Nevertheless the addition of certain substances to simple solutions give them certain properties of absorption which permit a comparatively considerable fixation of emanation.

Furthermore, the constitution of the vehicle should differ according to the absorption channel by which the emanation is to be presented. A vehicle for intravenous injection obviously must not have the same properties as the vehicle for local injections.

Hence the solutions which we have employed must be classified according to, principally: the solubility of the emanation, the channels of penetration. We have, therefore, (1) solutions with hardly soluble emanation, (2) solutions with medium soluble emanation, (3) solutions with very soluble emanation, and on the other hand: solutions for local injections, solutions for intravenous injections.

Emanation being an inert gas, is very slightly soluble in ordinary water and in aqueous crystalloid liquids (physiological solutions). Colloidal solutions will retain the emanation reasonably well; e.g., colloidal metals—organic colloids, blood serum. Fatty bodies will dissolve it almost entirely: paraffin oil, olive oil, vaseline, suspensions of liquid wax.

This is demonstrated by a simple and neat experiment. Let us take three ampules containing 10 cc. each of a liquid of the three groups mentioned above, and charged with 10 millicuries of radium emanation; and then observe them in the dark.

It will be seen that almost the total emanation is found in the empty part of the ampule containing the liquid series (1); this part will show the most vivid glow. In the ampule of the group (2), the luminosity is the reverse; the liquid shines more vividly than the empty portion. Finally, in the third ampule, the fatty liquid will shine almost exclusively.

Let us now examine the course of the emanation in the case of a living being injected with the vehicles mentioned above.

Doses Injected

The maximum emanation which it was possible to incorporate in a small quantity of liquid (1 to 10 cc.), was found to be 60 to 80 millicuries. The radioactive charge of the substance to be injected is estimated approximately in millicuries. Let us remark that this is the sole therapeutic application where the notation in millicuries is rational. We employ emanation and the totality of its radiation, only in cases of local applications which utilize the radiations almost completely. From this, it follows that in equal dosage the injection of emanation is more powerful than an application.

Experiments on Animals

Living Animal.—Introducing into the heart or the jugular of a female guinea-pig in gestation a certain dose of radio-active solution (2 to 4 millicuries

of radium emanation) and placing the animal in dorsal position on a photographic plate, we obtain, on exposing for different times, dissimilar images.

That obtained directly after the injection corresponds with the heart of the animal, characterized by its four cavities. Moreover, the image is double, meaning it depicts the two successive contractions of the heart (the guinea-pig had moved). The same phenomena has been observed during injection along the course of the vessels.

A photograph taken several hours later (one or two hours after the injection) shows a precise image of the embryos carried by the guinea-pig. It is possible to obtain analogous images, though much fainter, even 24 hours later. If the female litters, she no longer impresses the sensitive plate; on the other hand the newly born young produce images of variable intensity. Repeated experiments always show the predominant fixation of the emana· tion by the embryonic cells.

Let us remark that the endothelium of the vessels and of the heart do not fix the injected radio-active substance.

With a normal mouse, exposed 6 hours after injection of two millicuries of radium emanation, the plate shows nothing. With the animal killed 6 hours later, of the organs, only the stomach records an impression (it contains food).

Dead Animal: Experiments on Mice.—If the animal is killed immediately after the injection of 2 millicuries of radium emanation, the animal does not impress the photographic plate, but the separately exposed organs affect the plate with variable intensity, confirming the results obtained by previous authors. If the animal is killed one hour after the injection, the outlines of the animal are seen on the photographic plate.

Animals Carrying Tumors

Benign Tumors of the left ear; rabbit of 3½ kg. weight.

Dr. Itchikawa injected into the veins of this rabbit 20 millicuries of radium emanation, the animal carrying a fibroma of the size of a goose egg on the ear opposite the place of injection. A dental radiographic film placed on the fibroma one hour after the injection and left there for 4 hours showed no impression of any kind.

Malignant Tumors.—On the other hand, two rabbits carrying malignant tumors (established carcinoma), 14 months old, were given 15 to 20 millicuries of radium emanation. Under conditions exactly as in the case of the fibroma, the films gave a clear image and the outline of the tumors. With cancerous mice the same results are obtained. But if the mouse is killed 6 hours after the injection and its organs exposed, *only the tumor* gives a very clear image as compared with the ·faint stomach image.

Patients with Malignant Tumors.—Injecting 5 to 10 cc. of radio-active auto serum charged with 10 to 20 millicuries into the venous system of a patient suffering from a neoplasm, we observe a whole series of phenomena analogous to those seen in the case of animals.

At first, a sensitive plate placed on the course of the vein at a certain distance below the point of injection (e.g., 10 to 15 cm.), permits us to follow the passage of the radio-active substance. A few minutes after the injection a plate placed on the same place will remain without any impression of any kind. We shall again speak of this fact when discussing the theory of selective fixation.

After a certain time a photographic plate placed opposite the neoplasm will, after development, show an image exactly corresponding to the tumor; it shows the approximate volume and the probable shape.

In the case of cancers complicated by ganglionic metastases in the organs, the same experiment will make it possible to obtain a clear photographic image of the common or clinically presumed metastases.

CURIEGRAPHY

The images of embryos or tumors obtained in the case of the living animal are called Curiegraphs (A. Kotzareff).

We here reserve the term of Curiegraphy solely for the images obtained (after intravenous injection) by the selective fixation of radio-active substances in embryonic and cancerous cells in the case of the living being (man or animal). This method must not be confounded with the photography of hollow organs filled with an opaque substance charged with emanation, or with the photography of organs taken out after death and directly exposed on a photographic plate. This latter method may serve for the study of the degree of distribution of radio-active substances in various organs. It is of great importance in the treatment of cancer, especially for the cancerous metastases in the various organs.

There are two ideas to be borne in mind:

(1) The *curiegraphic image* of a malignant tumor obtained on a sensitive plate after intravenous injection of a radio-active substance. For this two essential conditions are necessary: (a) the animal must be alive; (b) the plate must be exposed at least one hour after the intravenous injection. Curiegraphy is only a method of diagnosis. No importance is to be attached to the intensity of the image obtained, as compared to the images made by the other organs (liver, kidneys, lungs, etc.) exposed directly on a plate but after the death of the animal; for these latter do not impress a sensitive plate during the life of the animal.

(2) *Organo-radiumgraphic or autoradiographic* image obtained of the different organs, especially the organs of secretion and elimination, through which the radio-active substance leaves the organism. But the organo-radiumgraph is obtained from the direct exposure on a sensitive plate of the organs concerned, i.e., after the death of the animal. This knowledge of the unequal distribution of the radio-active substances in the inner organs, is of capital importance in respect to the treatment of internal cancers or the metastases in the organs referred to. Consequently organo-radiumgraphy relates to internal curietherapy and is a therapeutic method.

This curiegraphic method is of great value in the diagnosis of neoplasms and especially of their metastases in the regions of difficult access for clinical exploration. Prior to the discovery of curiegraphy there existed no reliable process permitting verification of the existence of a presumed metastasis. Clinically and from statistics, we know the most frequent localisations of the various cancers; we may, with the aid of curiegraphy, preventively seek to discover the concealed metastasis. For instance, a cancer of the prostate will often cause a metastasis in the spinal column. We may take curiegraphs of the column at various levels which could indicate a heretofore unknown metastasis. We merely suggest this idea which we have not yet had opportunity to verify clinically. Besides, this injection will serve as anti-cancerous therapy.

The metastases of a tumor were not diagnosed until the time when they themselves developed morbid symptoms, and in this way, for a long time, they eluded efficacious therapy. Curiegraphy permits us to verify the existence of clinically suspected metastases, or, still better, to reveal in their very initial stages, metastases which would probably have remained concealed for a long time.

This process also offers the possibility of verifying the neoplastic nature of adenopathy. the exact nature of which is doubtful, and to make a confirmatory test after surgical intervention involving total or partial extirpation of a tumor-growth. Curiegraphy following treatment with a radio-active injection will very likely permit us to observe neoplastic recurrences *in situ*.

Before discussing the mechanism of the fixation of the emanation and trying to explain its selective nature, we will shortly review the different types of elective fixation known up to the present. We shall then see to which category the selective fixation of radio-active substances belongs.

CHEMICAL SELECTIVE FIXATIONS

From the chemical point of view it has been demonstrated that with animals, methylene blue injected subcutaneously will fix itself on the nerveends (Ehrlich). This fixation is compatible with the vitality of the cell.

Infusorians, rotifera, worms, and crustaceans can fix dyes only on their nucleus and continue to live and to subdivide (Przesmycki).

Another example of selective fixation is the staining of nuclear substances by basic aniline colors. These phenomena are explained by the combination of metaphosphoric acid with basic aniline colors, forming a precipitate of aniline metaphosphate. As we know that the albumin alone cannot fix the basic aniline dyes, the fixation is effected by combination of the dye with a phosphate substance (derived from nucleic acid), allied to nucleo-proteins (Vlès).

PHYSICAL FIXATIONS

From the point of view of physics, a simple example of fixation consists in the phenomenon of adsorption; an inert body, as for instance, animal charcoal, is capable of adsorbing large quantities of gas or organic liquids.

PHYSICO-CHEMICAL FIXATIONS

However, in the organism, the physical and chemical fixations are hardly ever found isolated; we nearly always face a combination of the two processes. It is physico-chemical fixation which determines all medical therapy and a great part of experimental physiology. We must remember that each cellular group has a chemical constitution of its own, which has been fixed in the course of philogenetic development and represents the chemical translation of morphologic cellular differentiation.

In accordance with the predominance of certain substances and their chemical affinities, a cellular group will fix certain substances of a definite chemical constitution, and will remain indifferent to others. These facts explain the action of our principal medicines (narcotics, analgesics, etc.), the symptomatology of many intoxications (plumbism, carbon monoxide, etc.).

BIOLOGICAL SELECTIVE FIXATION

Champy, amongst others, in studying thyroid extracts, demonstrated a cycle of absorption rather analogous to the one found by us in the case of

emanation: a rather general distribution of the substance at the start of the absorption, after which, and at variable periods, it fixes itself more and more on one system or organ. In fact, he states:

"Certain germinative zones (nervous system) are sensitive at the start, later, at the period of degeneration, their multiplications become slower. We thus reach the important conclusion, that, although some tissues actively react to the effect of thyroid extract, there are others which are completely passive in respect to it. The action of the thyroid principles, therefore, is plainly selective." Thyroid extract localizes itself finally on the pulmonary exterior.

Champy might have entitled his paper: "The action of the Thyroid Extract on Cellular Multiplication; *Selective Character* of this Action."

In the same line of thought we would mention the selective action of such substances as colloidal metals, homo- or hetero-albumins (the vitellin of the hen egg), trypan blue, trypaflavin, acids (Dustin, de Harwen) which induce kinetic waves either in the thymus or in the closed follicles of the digestive canal. A part of these substances is eliminated through the organs of secretion and excretion, but nevertheless we recognize the existence of selective action through fixation (by affinities of these substances).

Besides, of all the substances considered as organotrope (organophile) a part only produces a local selective fixation, as a predominant action, while the rest is eliminated as best it may; yet this does not prevent them being called substances of selective action (organotrope). We do not see any valid reason why we should not class radio-active substances as substances of selective fixation, because their action is not uniform and is influenced by the unequal distribution in the different organs.

Pathology offers numerous examples of selective fixation; tetanus toxin localizes itself in certain cells of the central nervous system, while the diphtheria toxin prefers the nerve-ends. In the living organism sensitized by foreign colloids (micro-organisms, vaccination, sero-therapy) specific, biological fixing agents are formed, of whose physico-chemical nature little is known. The Bordet-Gengou reaction is based upon this.

In the serum of an anaphylactized animal we observe an inversion of the micellary charges; at the same time in the case of the normal animal the electro-phoresis of the serum produces little precipitate at the negative pole, but much at the positive pole, while in the case of the anaphylactized animal the contrary phenomenon is produced (Kopaczewski).

In order to explain the different reasons for fixation observed in the organism, we must recall the physico-chemical laws regulating the relations of atoms, of the simplest molecules, amongst each other and with other elements. We are convinced that the same laws that govern inorganic matter also apply to "living" substances.

The forces which cause mineral molecules to approach or retreat are identical with those that act on organic molecules. In order to understand the inner nature of these forces which determine the grouping of organic molecules, and hence the phenomena of fixation, it is necessary to examine what happens between mineral atoms and molecules, in other terms, we must study physico-chemical affinity.

PHYSICO-CHEMICAL AFFINITY

It is to-day accepted that physico-chemical affinity is due to the attraction of atoms charged with opposite electricity (A. Berthoud). The inherent

electro-static energy of an atom is known under the name of positive or negative valency * and this valency, for the modern physicists and chemists, is merely a manifestation of energy depending upon the number and the charge of the electrons composing these atoms.

The atom is characterized by its electric charge and not by its atomic weight (J. Perrin); bismuth and lead derived from thorium have the same atomic weight of 208.

Positive valency represents the negative electrons capable of detaching themselves from the periphery of the atom to fix themselves on a positive ·atom; while negative valency represents positive atoms capable of fixing negative electrons.** On the other hand, the force which keeps the peripheral electrons bound to the atom depends primarily on the spherical charge of the central part of the atom, of the ion, or of the molecule. This central charge is represented by the number of the electrons furthest from the nucleus. By bombardment with X-rays electrons have been driven out of an element: carbon, potassium, selenium.

The identical forces which we have just considered in the physical chemistry of matter, are found again in the organic domain (humors, cells, etc.). The substances which constitute the organism are in a special state of physicochemical equilibrium, the colloidal state, which is characteristic of living matter. Each colloidal micell possesses, as do atoms and molecules, a characteristic positive or negative electric charge, depending upon the predominant charge of the micellary constituents (perimicellular atmosphere of the granule, or ionization of the micell).

Dissociation of Colloids and Precipitation

We know that an electric current passing through an electrolyte will separate the molecules into their constituent ions of opposite charges; the same current passing into a colloidal suspension will separate the micells of opposite charges which will accumulate at the opposite electrodes. For instance, the current passing through normal serum composed of micellary aggregates in electrostatic equilibrium, will lead the globulins to the positive pole (Lumière, R. Fischer, Kopaczewski).

In exactly the same manner acids (1% tannic acid) precipitate the globulins by neutralizing their electric charges.

Flocculation and Ripening of Micells. If a suspension (pseudo-solution) of a colloidal metal (e.g., colloidal gold) in which metallic particles (especially aluminium) have been distributed, is submitted to the action of X-rays, the flocculation and maturing of the metallic micells may be observed under the ultramicroscope. This flocculating action takes place in like manner in the case of suspensions or organic pseudo-solutions.

Under the influence of radiations the aluminium particles in their turn become centers of corpuscular and vibratory radiations, which discharge the micellary perigranular membranes that govern the colloidal equilibrium; the result is the flocculation, the ripening, and finally the precipitation of the

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* According to *Achame* the valency represents the excess of positive atomic charge. If the intratomic electrons neutralize all but one of the charges, the atom is univalent. It is bivalent, trivalent, etc., according as the number of unneutralized charges rises to two, three, etc. . . . ("La Molecule d'Hydrogène," 1925).
** If, for instance, we take chlorine and sodium, the atom of the former, on account of its tendency to complete its external rings, unites itself with the peripheral electron of the sodium. The 2 ions Cl and Na make more stable systems than two neutral atoms. Because of their electrostatic attraction . . . they combine themselves to a more stable molecule Na Cl. By similar reasoning the polyvalency of atoms is explained.

micells. The flocculation optimum is observed with homogeneous radiations; heterogeneous radiations, on the contrary, maintain the dispersion (Cluset and Kaufmann).

Radiations from different sources (X-rays, radium rays, secondary radiations, etc.) act on the humors or the cells of the organism and produce analogous phenomena.* We have shown that injected emanation restores the colloidal equilibrium of serum, and increases its dispersion: the flocculates of cancerous sera disappear (R. Fischer, A. Kotzareff). Perhaps we should recognize here the application of the stabilizing action of heterogeneous radiations, since we inject α, β, and γ rays of different nature and wave lengths.

We will now endeavor to explain the fixation of radio-active substances on the young and neoplastic cells by physico-chemical forces, based on the experimental facts reviewed above: we shall speak more especially of young neoplastic cells—they are of immediate and basic interest to us.

Emanation has an action upon cancerous autoserum; it *stabilizes* it. This is made evident by the fact that radio-active autoserum submitted to the electrophoresis, produces only an insignificant precipitate at the positive pole. The stabilization of cancerous autoserum is further confirmed by the Chodat reaction (tyrosinase-paracresol) and the Kotzareff reaction (precipitation by tannin). Let us recall that normal serum contains 5.24 per cent of positive albumin as against 2.40 per cent of negative globulin and that the radium emanation sends out 92 per cent of positive α rays as against 3.2 per cent of negative β rays, and 4.8 per cent γ rays. The positive rays although more numerous are the more ephemeral. They transform themselves rather rapidly in the course of their action, they are not very penetrative and their collisions with atoms produce negative corpuscular δ rays, by detaching electrons. From this it follows that the injections act by the negative β rays, the secondary negative γ and β rays, and the secondary negative δ rays.

The action of the α rays on a normal serum *in vitro* is perceptible and leads to its lability, while *in vivo* on the normal as well as on the cancerous animal, it is the phalanx of negative rays which act and stabilize. It will be understood that by saturation of the negative globulins by negative corpuscular β and δ rays, a more stable electro-colloidal equilibrium is produced. But the amount of emanation injected plays a direct and important rôle (small doses are exciting, medium doses *inhibitory,* strong doses have a necrotic effect, from the point of view of their action on the cells).

Radio-Active Substances in the Circulatory System. Once introduced in the blood stream, colloidal radio-active substances will act on the serum of the cancerous patient, in the same way as on his auto-serum, that is, it will stabilize the globulins and the albumins; the fact is proved by the different reactions of the humors obtained from one week to one month after the intravenous injection.**

The endothelial cells of the blood vessels, as we have seen, do not fix radio-active substances, which seems to demonstrate the physico-chemical indifference of their cellular walls (particularly of their colloids) with respect to the emanation.

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* It is possible that β rays inhibit the decomposition of organic matter (A. Boutaric, "La Vie des Atomes," Flammarion, 1923).
** This stabilization is so effective that the break-down of albumins into amines and amino acids, which is normal for cancerous serum, no longer takes place.

SELECTIVE FIXATION PROPERLY SO CALLED

The question here arises as to how radio-active substances behave in the presence of young cells and neoplastic components. The micells, vehicles of the absorbed emanation, complete their tour through the organism in 27 seconds and come into contact with the cellular walls with the interstitial liquid. As we have seen in our study of cellular membranes, the walls of young and neoplastic elements show a considerable diminution in their surface tension and a corresponding increase of their permeability.

The emanation absorbed in excess by the micells of the vehicle, partly abandons the micells to go to the cellular walls, which have more accentuated absorption and greater permeability. This phenomenon repeats itself at every passage of the blood stream into the vascular area which encloses a tumor or embryonic cells, so that one hour after the injection of the radio-active solution, it is possible to obtain curiegraphic images.[*]

The emanation absorbed by the cellular wall penetrates into the interior of the cell by diffusion, and proceeds to distribute itself by virtue of the different electric charges on the cellular colloids. These fix the emanation by adsorption and chemical affinity. However, even during the course of these phenomena the spontaneous disintegration of the emanation proceeds. The corpuscular positive α radiations fix themselves on the colloids of predominantly negative electric charges,[**] that is, the cytoplasmatic colloids which contain radio-active potassium of opposite charges send out negative β and γ rays. The corpuscular negative β rays proceed to fix themselves on the predominantly positively charged colloids, that is, the nucleus. Finally the γ radiations produce secondary radiations by striking metallic atoms.

The Proofs of Selective Fixation. To summarize, the selective fixation of radio-active substances on embryonic and neoplastic cells seems to us to be demonstrated by a series of facts, of which curiegraphy appears to us to be the most important. This fixation is also confirmed by the fact that tumors rich in mitosis before the injection show, one or two weeks later, less numerous mitoses and the nuclei on the way to degeneration (caryorhexis and caryolysis).

The negative serologic reaction after injection demonstrates indirectly the action of the emanation on the metabolism of the cancerous cells, and its repercussion on the humors.

Still more significant, an electroscope placed in the vicinity of a tumor which has fixed emanation, is discharged; while, after the interposition of a lead screen, or shifting of the apparatus, its leaves remain apart. Nobody will underestimate the considerable interest of this experiment for the diagnosis of neoplasms and their metastases; unfortunately we have only verified it a few times due to lack of patients, radio-active substances and material opportunity.

To conclude, we consider that the physico-chemical properties of young and neoplastic cells, and also those of radium emanation explain, at least theoretically, the question of the selective fixation of radio-active substances. The actual reality of curiegraphy is, in fact, indisputably proven.

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[*] The higher the permeability of the membranes, the better the chemical composition of the cell can enter into play. So Falta thinks that fixation of the emanation, which he calls tropism, is due to the presence of lipoids.

[**] The micells have normally negative charges in the immense majority of liquids and tissues. This very slight electronegativity should have great significance for the affinity of the substances dissolved in the blood plasma. It should influence those chemical reactions which happen in the interior and on the surface of the protoplasmic micells, and finally determine the action on the living cells of external factors (J. Verne).

The Filtrable Entity Transmitting Chicken Sarcoma

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The question of the filtrable entity transmitting chicken sarcomas has a special significance in cancer research, for if the chicken sarcoma, an apparent true neoplasm, is transmitted by a filtrable "agent," other animal and human tumors may also be associated with a similar agent.

The true nature of the filtrable entity is yet undeterminable, despite the large amount of work that has been done on the subject. There is also room for doubt as to how completely that entity can be separated from the sarcoma cells. Inasmuch as the methods employed in the investigation, namely, filtration, centrifugation, etc., involve delicate physico-chemical questions, the subject may offer many problems for study from the standpoint of colloid chemistry. It is essentially for this reason that this brief account is presented here.

FILTRATION

Up to the present the sole means of determining the filterability of the so-called agent consists of the sarcoma transmission test by injection of filtrates into chickens. By this means it was early shown by Rous and Murphy [1] that in a dilute tumor extract in Ringer solution the "agent" is capable of passing through Berkefeld filters impermeable at the same filtration to *Bacillus fluorescens liquifaciens,* which measures 0.5 micron by 1.0 to 1.5 microns. It passes most V cylinders, many designated as N, but is usually retained by the fine textured W cylinders; points confirmed by Fujinami,[2] Hayashi,[3] etc. Recently Gye and Andrews [4] offered the following summary of the filterability of the Rous sarcoma.

Paper-pulp and sand filter ....Filtrate exceedingly virulent.
Chamberland L 1..................Also very virulent but much less so than the above.
Chamberland L 2..................A further loss of virulence.
Chamberland L 3..................Rarely "infective."
Chamberland L 5..................Innocuous.
Mandler filter ....Almost as virulent as Chamberland L 1.

Teutschländer [5] carried out a series of experiments with membrane filters which withhold particles of gold hydrosol of 0.000,001 mm. in diameter, and reported the following results:

Filters I and V, permeable to both *B. prodigiosus* and the sarcoma "agent."
Filters X and XX, impermeable to *prodigiosus,* but permeable to the sarcoma "agent."
Filters XXX and XV, impermeable to both *prodigiosus* and the "agent."

In later experiments Teutschländer [6] used the Zsigmondy-Bachmann membrane filter and stated that the minimum diameter of the pore permitting the passage of the sarcoma "agent" was in the vicinity of 0.6 micron. Ono [7] also obtained similar results.

It has long been known that the filtrability of the Rous sarcoma is very variable. Rous and Murphy [8] attribute this to the narrow limits within which the "agent" is filterable. Gye and Andrews,[4] finding that the filterability may suddenly appear and disappear during the propagation of this tumor, suggest that the filterability may not be its essential property.

The interpretation of the results of filtration experiment requires many precautions. The filtrability of particles of a given size is due not so much to their size as to the amount and kind of electric charge on them in comparison with the charge of the filtering medium. Alteration of the pH may greatly modify the charges of particles and of filter. Salt ratios, salt antagonism, and factors causing surface film formation must also be considered, and, in the case of living forms, the viscosity of protoplasm, and influence thereon of a great many factors. Obviously a satisfactory explanation of the variation of the filterability of the sarcoma "agent" awaits further studies on the physico-chemical processes involved.

THE FILTRABILITY OF SARCOMA CELLS

According to Fischer [9] the malignant cells of the Rous chicken sarcoma cultivated *in vitro* vary from the size of a large bacteria to several hundred times that size, and in fixed sections it is easy to find many cells as small as 1.5 x 1.5 to 2.5 microns. Under favorable conditions some of these cells may pass through a filter. Ogata and Ishibashi [10] had described "minute bodies, often showing nuclear staining," in Berkefeld filtrates. Teutschländer [6] reported that minute cells and cell-like structures can be found in the filtrate through a Zsigmondy-Bachmann membrane filter. A recent report by Harkins, Schamberg and Kolmer [11] stated that filtrates passing through the paper-pulp and sand filter show "from one to four cells per microscopic field (16 mm. objective)."

My experiments with Berkefeld filters (V and N) show that sarcoma cells, some as large as 2.5 × 3 microns may be found in the smear preparation of sarcoma filtrates (Nakahara).[12] The filters were proved to be bacteria tight by previous or simultaneous tests with *Bacillus prodigiosus*. The cells have round or oblong well-stained nuclei, which is often surrounded by a small halo. The cytoplasm stains pale blue with Giemsa solution and usually shows what appear to be minute ameboid processes.

It must be remembered that a centrifugation at high speed of saturated sodium sulfate solution can produce crystallization in the bottom of the tube.* On the other hand, there is great difficulty in separating all the sarcoma cells by centrifugation. In my experience, centrifugation of sarcoma cell suspension invariably gave rise to a top layer rich in cells, though naturally the majority of the cells were thrown down to the bottom. Under ordinary conditions it may be impossible to obtain a complete separation of the cells by centrifugation.

CURRENT THEORIES REGARDING THE NATURE OF THE FILTRABLE ENTITY

Rous and Murphy [1] showed that the sarcoma tissue retains its transmissibility when dried or preserved in glycerin, though undergoing gradual attenuation. When the tumor tissue is heated at 55° C. for fifteen minutes its transmissibility is completely lost. Most bactericidal substances rapidly destroy the transmissibility. The microbic nature of the agent cannot be demonstrated in the absence of successful passage in cultures, but Rous has regarded the "agent" as a living virus.

Gye,[13] in his recent experiments, advocated the existence of two factors as the cause of malignant disease. He showed that chloroform in a certain proportion renders the filtrate innocuous but that it can be reactivated by the addition of anaerobic cultures of chicken sarcoma or of tumors of other animals. He believes that in the filtrate of chicken sarcoma there are two necessary factors for tumor production: living virus and specific factor, of which the virus alone is killed by chloroform, while the addition of tumor cultures restores the virus and thus the mixture regains its original tumor-producing action. The fundamental assumption is that the virus is common to all malignant tumors, regardless of animal species. The subsequent work of Murphy [14] and Flue [15] show that "cultures" of normal tissue can likewise reactivate the sarcoma filtrate rendered innocuous by chloroform treatment. Moreover, evidence is growing to show that the tumor-producing entity in the original filtrate is single, and not dual as suggested by Gye.

The possibility that the filtrable agent might be a chemical substance has not been neglected. On the basis of some *in vitro* experiments Carrel [16] has elaborated upon this view. He assumes the agent in the Rous sarcoma to be a substance produced within the sarcoma cell, and capable of transforming normal tissue cells (macrophages) into malignant cells, which in turn produce the same substance. Carrel [17] experimentally produced several sarcomas in chickens by means of coal tar, arsenious acid or indole injections, and found that most of them can be transmitted by their filtrates.

Certain fundamental points in the transmission of the sarcoma, especially the high degree of cellular specificity manifested, are difficult to explain on the basis of either virus or chemical theory. Since the only known mechanism which is capable of perpetuating a specific type of cellular growth is the one expressed by Virchow, *"omnis cellula e cellula,"* it would seem necessary to keep in mind the possibility of the sarcoma cells themselves being in reality the transmitting agent, even when the participation of the cell is to all

appearances excluded. Obviously the process involved is not a matter of mere growth stimulation. It is even more complicated than a malignant transformation of affected normal tissues, as in the case of the tar tumor.

One of the fundamental problems that confront us in an investigation of this nature is the extreme difficulty of completely removing what we intend to remove by filtration and centrifuging. The factors entering into these processes are very complex and it is likely that further investigation of the question of the filtrable entity will take us into the realm of colloid chemistry.

<div align="center">NOTE BY EDITOR.</div>

In Chapter XIV on "Filtration Experiments," of "Colloids and the Ultramicroscope," Prof. R. Zsigmondy says:

"All three kinds of filters (Maassen, Pukall and Chamberland) contain pores large enough to allow the passage of gold particles of about 30 $\mu\mu$ and less. The pores of a cell are of very different sizes, the Chamberland cell containing, for example, large pores, which allow the gold particles to pass through, and others which retain most of them. The size of the pores is, however, not the sole criterion in filter experiments. It is of especial importance in coarse filters, whether the particles to be filtered are held to the surface of the cell by adhesion or "adsorption" (a), or not (b).

(a) In the first instance the substance to be filtered gathers upon the outside surface (and to a certain extent in the deeper pores), and prevents the other particles from forcing their way through; first, because the pores are made smaller; second, because the particles held fast to the surface of the cell repel the freely moving particles following the course of the current. This action may be due to the well-known negative electric charge of the particles, which apparently also affects the adhering gold particles.

(b) When adhesion or adsorption does not take place, all colloidally dissolved substances pass freely through the cell, providing the pores are large enough.

In the presence of a protective colloid, e.g., egg albumen, all the gold particles pass smoothly through, whereas in the absence of protectors, matters proceed as in case (a). The fact that protected gold particles of 30 $\mu\mu$ and over easily pass through Maassen and Pukall filters should be of interest to bacteriologists. The Chamberland filter, too, contains, besides the very small pores chiefly present, others which permit the passage of particles of the size mentioned."

Another point of great importance to bacteriologists has been emphasized by Professor H. Bechhold, who found that lecithin emulsions whose droplets were several μ in diameter passed through ultrafilters capable of retaining hemoglobin, and whose pores were less than 30 $m\mu$ (pressure 150 g./cm.^{2}). Bechhold explains that the droplets assume a filiform shape in their passage, reforming on their exit. (See Bechhold's "Colloids in Biology and Medicine," Bullowa's translation, Van Nostrand; also Vol. I of Alexander's "Colloid Chemistry, Theoretical and Applied," Chem. Cat. Co., 1926, articles on Ultrafiltration and Electro-ultrafiltration by Bechhold.)

Bechhold says in the latter reference, p. 832:

"Therefore the diameter of the pores of the ultrafilter gives no definite idea of the diameter of a retained particle as far as *emulsions* are concerned, whose disperse phase has a low surface tension against the dispersing phase."

Since the work of Heilbrunn, Chambers, Seifriz and others shows the great changes in viscosity which organisms exhibit during mitosis, and since changes in the milieu may produce similar changes, we must observe many precautions before hazarding an opinion about size deduced from filtration experiments. Alteration of the pH of the milieu may modify the charges of particle and of filter, and even reverse them. Salt ratios and antagonism must be considered, as well as anything leading to formation of surface films. And these or other factors may influence the viscosity of protoplasm. Professor H. Schade * illustrates a phagocyte passing in filiform fashion through an orifice very much less than its average diameter, and appearing in its usual guise after it emerges on the other side of the membrane.—*J. A.*

* See his paper, this volume.

Some Therapeutic Measures Founded Upon the Colloid Theory of Malignant Tumor

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The principles explained in a foregoing paper [1] should prove of value in developing means of curing malignant growths. While it may be stated that such an ideal is still far from realization, nevertheless some experiments in this direction might be described.

In the theoretical paper, one of the most essential points was the considerable influence exerted by bivalent ions on malignant tissue, an influence whereby, e.g., a normal permeability, normal electrical behavior could be restored.

This naturally leads us to examine how malignant tissue *in vivo* and *in situ* is affected by *different* bivalent ions.

Of course in our theoretical discussion, we referred in general to "bivalent ions"; practical use was made almost exclusively of "Ca" and "Mg" ions, the normal physiological bivalent ions. In therapeutic measures other bivalent ions must be studied more closely.

While all bivalent ions have in common their resistance-increasing action on the malignant tissue, there exist of course differences, due to different *chemical* properties.

One difference exists initially, in the *reversibility* of the action of different ions, which, with "Mg" and "Ca," is totally reversible and may be counterbalanced again by monovalent Na and K. Other bivalent metals, Cd″, Zn″ and Pb″, exert a more lasting influence.[*] Next to their electrical influence, their chemical behavior and the stability of their combination with proteins and soaps come into play.

It may be interesting to describe briefly the different changes which malignant and normal tissues undergo *in vivo* under the influence of the different ions.

I have made studies for a considerable time, and Borrel [2] in Strassburg reported experiments in the same direction.

TECHNICAL WORK

The advancement made in experimental tar carcinoma of the skin permits a close study of the various changes effected by the different procedures, far more accurately than in the case of subcutaneous transplantations.

[1] Waterman, *Biochem. Z.*, 133 (1922).
[*] Here is a case where ions of the *same* valency exert different effects. See footnote in preceding paper. *J. A.*
[2] Borrel, *Compt. rend. Soc. Biol.*, 81, 208 (1922).

The problem is simply to treat the skin with different ions. Therefore a piece of cotton wool or blotting paper is soaked in isotonic concentrations of the different salts, and the positive pole of a galvanic circuit is put in contact with it. The negative pole is on the opposite part of the body and fixed to it in the same way with electrodes soaked in physiological salt solution.

The current used may vary between certain limits. Ordinarily a current of 10 milliamperes was used, with a voltage of 40-50.

The duration of the experiment varied from 15-30 minutes. From these data the electrochemical equivalent can easily be calculated. The amperage has to be regulated with a variable resistance, for as a rule the amperage varies during the treatment with bivalent ions. As a rule the resistance *increases*, but it must be admitted that it is difficult to prove that this phenomenon is real, because the movements of the animal may account also for changes in the resistance.

The effects of the treatment with the different bivalent ions (Ca″, Mg″, Cd″, Zn″, Pb″) demonstrate that all ions show the same influence, differing only quantitatively and in duration.

Fig. 1.

The photographs (Figs. 1 and 2) show the classical difference between mono- and bivalent ions *in vivo*. Here the effects of K, Na, and Ca ions are compared after a treatment of 15 minutes. It is clear, that while skin treated with the monovalent ions shows only very slight, if any, changes, the influence of the bivalent Ca is marked.

These changes may be examined both macroscopically and microscopically. The first effect after treatment is the appearance of a white spot on the part where the electrodes have been attached, with some slight blood red extravasations, a proof that blood-vessels have been injured.

The next day, the white spot slowly becomes brown, and gradually dries into a crusty mass. At the end of several weeks, the brown crust is stripped off and the underlying healthy granular tissue slowly is covered by new epithelium.

The microscope reveals the different stages of the process far better; in short, we see the different stages of necrobiosis in the upper layers of epithelium; under these, different degrees of desiccation of the various cell groups are apparent. Very remarkable is the injury and calcification of the muscle layer, while the outer border of the whole process consists of a large area of inflammatory tissue (lymphocytes, plasma cells and newly formed spindle cells).

In various stages various processes prevail, while at the end the tissue is replaced by a new healthy one. The calcified strata remain, however.

We must emphasize here now, that the action of the bivalent ions studied is different only quantitatively, and increases in the order already stated:

$$Mg < Ca < Zn - Cd < Pb.$$
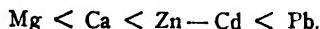
The changes thus far described seem in good agreement with what we could expect on the basis of our former theoretical explanations. Still, it must be confessed, that control experiments leave some doubt as to whether our observations are in perfect harmony with the theoretical assumptions.

FIG. 2.

As in all cases the chlorides of the metals were used, the influence of the anion may be neglected.[*] It seemed desirable, however, to see if the effects described were to be attributed wholly to the metal *ion*.

Therefore some experiments were performed at the same time with the Mg″ ion as such, in chloride solutions, as well as with solutions of Mg citrate, where there dissociates not the Mg ion, but another ion-complex, namely:

$$\left. \begin{array}{l} Mg_2Ci\ + \\ MgCi\ - \end{array} \right\}$$
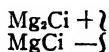
Some difficulties in preparing isoionic solutions of this kind were encountered, besides which it is not certain that the pH of the solutions used was exactly the same. Neglecting these objections for a moment, the results with *all* the Mg solutions were the same. We are lacking therefore *absolute* proof that our explanation, based on our preceding model-experiments, is right.

We must describe now the reaction of *malignant* tissues of the skin, treated in the same way. The phenomena observed may be briefly stated as follows:

[*] As dissociation is variable, this seems questionable. *J. A.*

a. The action of bivalent ions is, approximately, the same as described for normal tissues, but far more powerful. In addition (and this is very characteristic), the effects described pass away much quicker, so that the state of crust-formation is reached much earlier.

The action of the heavy metal-ions is, here too, far more intensive than those of Mg and Ca", while the *Pb"* *ion* exerts the most powerful and lasting influence.

It is possible, then, to cause the disappearance in this way, chiefly by Pb, of papillomata and *carcinomata which are not too far developed and extended.*

The full microscopical details must be dealt with in a later publication. In larger carcinomata total recovery is only very seldom attained, though often partial healing occurs. These results are not quite comparable with those of Borrel, for the treated malignant tissues are very different in both sets of experiments: in Borrel's experiments, transplanted, poorly fed tissues; by me, autogenous and very malignant, metastasis-forming carcinoma was treated.

b. Another phenomenon attracts attention. Next to the *"reinstutio ad integrum,"* which is to be observed in the way described, it is found that in many cases recurrences in the area of the treated part of the skin are frequent; not only do they arise in the very bottom of the healed spot, but at its whole boundary. Further the impression forces itself on one, that development of new papillomata and carcinomata at a further distance is more frequent in the treated cases. This is only an impression, for one can never tell at what new spot a new papilloma or carcinoma will arise in a tar-treated mouse. The only explanation, therefore, is that in various spots the apparently healthy cells are irritated by these different measures and their consequences, and that the apparently healthy epithelial cells are not at all normal, but are already in a state of invisible disturbance. Every new irritation, mechanical (scarification), chemical, or inflammatory, may cause the development of new malignant tissue. It is especially remarkable that, on totally extirpating the original spot, new tumor tissue may develop just where the sutures were made. These observations speak for a general cancerization of all the animal cells. At the same time when the investigations described in this and the first paper were performed, therapeutic measures were employed in England by Blair Bell,[3] on similar lines.

Blair Bell observed the prominent effects of lead in injuring life-processes, and attributed this to the solubility of the metal in lipoids, chiefly because of affinity to the phosphatids of the cell-membrane. In our first paper we have dealt largely with the function of the cell interface in explaining the electric peculiarities of the malignant cell.

What Borrel and the writer tried to do by external *ionophoresis* in experimental carcinoma, Blair Bell[3] applied to the treatment of internal carcinoma by intravenous injection.

The great difficulty was to find a stable colloidal lead preparation, with sufficient metallic activity. After many combinations, e.g., with lecithin, ordinary colloidal lead, obtained by the classical method of electrical dispersion (Bredig),[*] proved the most satisfactory. From some analytical data it seems, indeed, that the theoretical basis is sound, as the metal is deposited in the

tumor-tissue to a large extent.* The table shows some data obtained from a human autopsy, following lead treatment.

Organ	Lead in Mg. per Cent of Fresh Tissue
1. Liver	2.54
2. Spleen	5.83
3. Lung	0.52
4. Kidney	0.77
5. Uterus	0.47
6. Bone	0.58
7. Bone tumor (carcinoma)	10.2*

It seems therefore that it may be possible to treat some forms of tumor in this way. The procedure, however, is as yet in an experimental stage. What should make one very sceptical and cautious is, that formerly advocated methods of treatment of the same kind (e.g., the selenium compounds of Wassermann, heavy metal combinations of Neuberg) have proved unsuccessful with *human* malignant disease.

* The work of Aub and others at Harvard University indicates that on exposure to lead, the metal tends to be stored in the bones as insoluble lead phosphate. There is such a close line between the therapeutically active and the poisonous dose of lead, that extreme caution must be used in experimental treatments. Lead preparations readily undergo changes after preparation, as well as after administration. *J. A.*

Colloidobiological Study of the Vitamins

By Dr. F.-V. von Hahn,*

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Investigation of the character of those unknown substances which C. Funk has called vitamins, E. Abderhalden accessory food factors, and R. Berg complettins, has hitherto been attacked from the most different angles. At first there were clinical observations which indicated that lack of some factor in the diet was responsible for certain pathological changes in the human and in the animal body. In the course of time it was thought that four diseases could be recognized as "nutritional diseases of the avitamin type," namely, rickets, beri-beri, scurvy, and xerophthalmia. It was logical to attribute the outbreak of an illness to lack of a constituent in the diet. These "vitamins" were designated by letters: A protected against rickets, B against beri-beri, C against scurvy, E against xerophthalmia; D stood for the vitamin which catalyzes the growth of yeast.**

We will not consider the whole useless and confused renaming, according to which vitamins A and E exchange their rôles.

Funk's thesis is this: that the avitaminoses mentioned are diseases caused by lack of a vital factor in nutrition, and cured only by the addition of this factor to the faulty diet. If rickets is considered from this aspect, it may be seen that this disorder is not to be conceived of as a "nutritional disease of the avitamin type." Diseases of bone development in children may be cured at least as well by irradiation as by changes in the diet. Quite apart from the fact that the action of cod liver oil, the most valued remedy for the English disease, may be explained without assuming it to contain a certain substance, vitamin A, rickets is not to be considered a pure avitaminosis because of the possible therapeutic effect of the sun, ultra-violet rays, etc. For this reason the reactions of that fat-soluble vitamin will not be taken up in the course of the discussion below.

There is too little exact experimental material for judging the other fat-soluble vitamin, lack of which causes the symptom complex of xerophthalmia, so that we must refrain from considering this "vitamin" also from a colloidobiological standpoint.

There remain, therefore, for further discussion, which will be developed herein, the three water-soluble vitamins, B, C, and D, or as otherwise called, the anti-beri-beri vitamin, the anti-scorbutic vitamin, and the catalyst for the growth of saccharomycetes, which is apparently identical with Wildier's substance called "Bios."

According to the previous conception, which is influenced principally by Funk's thesis, the three vitamins must be three different chemical substances. Each vitamin must be a different chemical compound, which would be the same in all naturally occurring remedies for the disease. Two questions arise whose solution is particularly important. (1) What is the evidence for and against the view that vitamin B, or C, or D is each a definite therefore specific substance; and (2) what is the evidence for and against the opinion that the three water-soluble vitamins named are identical? (The first question may be formulated differently: Are there chemically definable vitamins?) According to our opinion, two groups of experimental findings oppose the existence of a vitamin which can be characterized by a chemical formula: on one hand the behavior of the hypothetical vitamin toward physical agents; on the other, the cure of avitaminoses by artificial, i.e., synthetic preparations.

"We do not yet know," writes Funk, "whether . . . we are concerned with the same or different substances, for this varying behavior toward external agents is not necessarily to be attributed to the presence of different substances. . . . For it is indeed possible that this may be governed by the different kinds of combinations of the vitamin and by the properties of the liquid." This statement applies to the so-called vitamin C. To give only a few examples of this: dried white cabbage is potent against scurvy, dried dandelion is not; in fresh condition the activity of both is the same. Juice of white cabbage loses its prophylactic properties on drying; lemon juice in tablet form retains its potency a very long time. The anti-scorbutic effect of potatoes, carrots, etc., disappears on cooking;* lemon juice remains active even after an hour's heating at 110° C., etc. The stability of this vitamin was attributed by Holst and Frölich to its acid content; experimental preservation of anti-scorbutic potency in other thermolabile substances by acidifying, was not, however, completely successful; since 1912, the year of publication of the work of Holst and Frölich, no further advance has been made in this field.

One of the weightiest reasons against the chemical homogeneity of "the" vitamin B or C, is the possibility of affecting the development of the so-called avitaminoses by known chemical compounds. It is a singular attitude, fortunately seldom appearing in scientific literature, which Funk takes toward work planned for the advance of knowledge in this field. The statements with which he estimates the investigations which attack the dogma of specificity of vitamin action, may follow here because of their peculiarity:

"A short time after the publication of our chemical studies of the vitamin, we found a whole series of investigations which reported curative results in beri-beri in fowls following use of wholly different, heterogeneous substances. These reports had the undesirable result of casting doubt upon the specificity of vitamin action. It almost seemed as though substances of nearly all chemical groups could function as vitamins." To describe as "undesirable result" the effect of researches published independently by authors such as Abderhalden, Dutcher, McCullom, etc., authorities in the field of nutritional physiology, is a singularly one-sided stand, which should be opposed most strongly. The cure was explained by Funk as due in part to the mobilization of vitamins present in the organism; although Funk himself, in accord with the experiments of Hess, is of the opinion that there are no reserves of vitamin C in the body, and that all vitamins, except B, which is also potent parenterally, must be given orally.* Funk's less cogent arguments against the

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* This naturally depends on time and temperature of cooking. *J. A.*
* Ergosterol can be activated by ultra-violet rays *after* being introduced into the body. *J. A.*

"vitamine action" of pharmacological preparations will be referred to with the individual experiments.

In reference to the relation between vitamin action and intestinal function, there are experiments of McCullom and Pitz, who found that laxatives such as mineral oil, phenolphthalein, and others, exert a curative effect on scurvy.

Unfortunately without further evidence, Funk writes, "The views of Mc Cullom and his co-workers, as we may well understand, have raised a storm of protest." As will be brought out in a later publication, we also have observed a favorable influence on avitaminoses with surface-active laxatives. We cannot agree, however, with McCullom's opinion, that the vitamin potency of fruits, etc., depends upon their aperient action, since, as is shown below, we consider another property, common to all active material, the cause of the healing effect.

Abderhalden and Lampe stated that castor oil exerts healing action on beri-beri; in a later communication Abderhalden and Ewald were unable to confirm this statement. As different preparations of castor oil produce strong, varying surface activity in the chyme, in our opinion the reaction is variable. Interesting investigations in relation to alcohol have been made in experimental and natural avitaminoses. It is remarkable that as early as 1757 Lind found that next to oranges and lemons, cider was the most powerful remedy in scurvy. Osborne and Mendel showed that the vitamin content of apples is apparently small. There is strong probability that alcohol is potent; particularly, the presence of traces of yeast is not to be overlooked. This becomes more apparent from the experiments of Miyadere, who attacked the problem of why the poorer classes of Japanese, who live almost exclusively on husked rice, do not develop beri-beri; he came to the conclusion that the rice wine, which contains 12 per cent alcohol and is drunk before and after meals, exerts a prophylactic effect.*

In a painstaking investigation he studied the secretion of gastric juice in a dog on a vitamin-free ration and found the flow completely checked on a long continued diet without vitamins. We are not of the opinion that this is a primary result; resorption must take place in the normal way, but according to our experiments would be affected by giving alcohol. Interesting contributions to the question of the connection between alcohol consumption and avitaminoses are found in old nautical stories, as well as in medical treatises of sanitary conditions on Polar expeditions. As was again observed in the Russian scurvy epidemic, the disease often breaks out when the supply of spirits is low. By a collection of histories of scurvy cases, attention should be drawn to alcohol consumption, even when it is given only in limited quantities.

Additional compounds, which may have a curative effect on avitaminoses are, for instance, adenine.* This is a 6-amino purine of the structural formula:

$$
\begin{array}{c}
\text{N} = \text{CNH}_2 \\
| \quad\quad | \\
\text{HC} \quad \text{C} - \text{NH} \\
\| \quad\quad \| \quad\quad \rangle\text{CH} \\
\text{N} - \text{C} - \text{N}
\end{array}
$$

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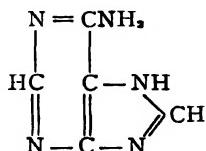
* Possibly small but potent traces of the products of yeast metabolism or lysis are effective here. *J. A.*

* Many do not recognize the curative effect of adenine. Its transformation into an active form referred to just below, recalls the behavior of ergosterol. *J. A.*

It belongs, therefore, to the purine group and is related to uric acid, as well as to caffeine, theobromine, theophylline, etc. On condensation, Seidell and Williams obtained a therapeutically active isomeric modification, whose potency disappears on conversion into the usual adenine by recrystallization. With the possibility of considering adenine, as above shown as 6-amino-purine, or also as 6-imino purine 1.6 dihydride,

$$
\begin{array}{cc}
HN-CNH & \\
| \quad | & \\
HC \quad C-NH & \\
\| \quad \| \quad \rangle CH \\
N-C-N &
\end{array}
$$
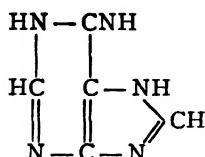
this report merits consideration, though Voghtlin and White could not confirm it; more recent experiments, which will be reported later, show considerable potency in caffein similarly used. Harden and Zilva tried to obtain an active product from *o*-oxypyridine and adenine. Later Williams reported the curative action of β-oxypyridine, β-methylpyridone, and 4-phenyliso-cytosine against beri-beri. Funk considers that Williams' animal experiments were not very convincing. According to Funk, several purine and pyrimidine derivatives show a distinct life prolonging action; Williams and Seidell were able to confirm this. Another organic substance which is potent against beri-beri is β-imidazolethylamine which Abderhalden and Ewald found active. In addition, a series of alkaloids should be named; Cooper found a distinct action against experimental beri-beri in quinine and strychnine; later he explained the effect of quinine by vitamin contamination of his preparation. Here is an interesting case where an author's first work must be defended against his later statements; as will be reported later, we also were able to confirm the potency of quinine which had been repeatedly purified. The action of strychnine was explained by its usual effect on the metabolism; but to distribute the effect between general and vitamin action is, we think, only a matter of definition. According to Dutcher, pilocarpine is active; this is disputed by Abderhalden and Ewald.

Uhlman found that the pharmacologic action of pilocarpine and choline is similar to, if not identical with, that of orypan, which indisputably is an excellent remedy for beri-beri, and with that of several vegetable extracts. Furthermore Dutcher claims that thyroxin and tethelin exert a healing influence on beri-beri. Vedder and Clarc found no potency in thyroid preparations; Seaman detected vitamin B in them.

The second question which we postulated reads: Have identical substances been hitherto considered as different chemical compounds? In other words: Is, perhaps, the lack of one and the same substance the underlying cause of beri-beri and of scurvy? Several reasons make it seem probable that this is so. Indeed it appears from a consideration of clinical findings, as well as from metabolism experiments, that the symptoms of the so-called avitaminoses have great similarity to those of starvation. We think that the reason most authors take separate etiologic instants for the outbreak of scurvy and of beri-beri, is to be sought in three directions: first in the neglect of clinical observations, which often present mixed forms ("Mischformen") of both diseases; next in too great dependence on animal experiments; and finally, in faulty attention to proportion in the so-called vitamin-containing foods.

With regard to the neglect of clinical evidence, which certainly is obtained in confused, not in classic or homogeneous pictures, we may refer to Holst's publications, which report the development of scurvy symptoms in pellagra; loss of hair particularly and porosity of the bones are peculiar to both diseases.

In professional reports from daily papers concerning health conditions on Polar expeditions, the confused nature of the diseases is often evident; thus G. v. Wendt, in a report states: "The crew . . . had one sickness which was not quite pure beri-beri, but resembled a mild scurvy." Often those who have the opportunity to see scurvy clinically, will find symptoms which would be described as characteristic for beri-beri, especially such as the so-called atrophic forms (muscle atrophy, equinus position, etc.).

In experimental avitaminoses in animals, different transitions from beri-beri to scurvy have been described. There is a striking report of Hume and Tozer, according to which guinea pigs, which were fed a vitamin A free ration, developed an osseous condition which was very similar to that in scurvy. It is particularly remarkable and noteworthy in how many cases of experimental beri-beri, symptoms appear which are identical with those of prolonged starvation. Funk defines beri-beri as a "very definite retardation of life processes similar to that observed in starvation." He quotes as in agreement with this, the observations of subnormal temperature of Drummond and Abderhalden. Lumière describes the occurrence of beri-beri on a diet containing vitamin, but quantitatively insufficient; Funk suggests the possibility "that here hunger weakness was confused with beri-beri." Also the decreased catalysis in the organs of rice-fed pigeons, which Dutcher and Dutcher and Collatz observed, was reported in almost the same way in starving animals by Burge and Neill. We will discuss later the significance of the close similarity of beri-beri (and in certain instances also of scurvy) to starvation symptoms.

In our opinion the evidence of animal experimentation in the study of avitaminoses has had very important results in estimating the specificity of the vitamins. It is usually customary, and is always emphasized by competent authors, as Funk and others, that scurvy is studied most suitably in rats * and guinea pigs, and beri-beri in pigeons. In such cases can the two diseases be compared at all? If one considers how differently scurvy, for instance, appears in young and growing individuals, it is not to be wondered at that in different kinds of animals one and the same disease can show different symptoms—in birds, chiefly the beri-beri condition of polyneuritis; in rodents, scurvy.

From the tabulation of foods according to their vitamin content, which Funk has so excellently prepared, it appears that every food that is antiscorbutic also contains vitamin B. Very few of the foods containing vitamin B do not contain vitamin C; there are, however, several which protect pigeons from beri-beri, but produce scurvy in rats if fed exclusively; they are, however, essentially such foods as are not the natural diet for rats, particularly fresh green vegetables. It is therefore possible, after all, to attribute the inactivity of the substance to the fact that the kind of animal chosen does not assimilate the diet.

To this it may be opposed that in humans there occur two separate diseases, scurvy and beri-beri, that therefore there must be separate underlying etiological moments. This conclusion is not justified. If we assume that the

* Scurvy is never studied in rats. *J. A.*

vitamins act by enabling the cells of the intestinal tract to resorb the food contained in the chyme—and this working hypothesis we expect to be able to establish by experimental evidence to be offered in later publications—it depends chiefly in resorption diseases (and as such we consider the avitaminoses) upon what substances are available in the chyme. It is known that hunger symptoms differ in quantitatively insufficient, and in wholly deficient diets, according to what is given the animal. If the vitamin insufficiency in humans is only that of rice and similar products, the picture of beri-beri develops; on another, more varied diet, which is nevertheless free from fresh vegetables, symptoms of scurvy appear. It does not follow from difference in diseases that lack of different vitamins is the cause; deficiency in one factor in combination with different kinds of basic diets may be the reason for the apparently heterogeneous symptom complexes of scurvy and beri-beri.

It follows from the work of Harrow and Krasnow * that the hypothetical vitamins B and C are closely related; in germinating seeds vitamin C develops at the expense of vitamin B, which in resting seeds is present in greater amount; indeed, according to other reports, occurs alone. The experiments are explained easily by assuming the identity of both vitamins. As to the possibility of the formation of vitamin C from the B of the diet, Funk is in accord with the researches of Parsons, who reported that the organs of rats contained large quantities of vitamin C even if they were fed a scurvy producing diet.

On the basis of the evidence submitted, it appears in answer to both the above postulated questions, that the water-soluble vitamins B and C can be identical—further, that nothing supports the idea that *the* vitamin is a chemically definite substance.

3. This answer contains a contradiction, for if there is no single vitamin substance, how can one speak of identity? We next took as our working hypothesis, that there is no vitamin-*substance,* but rather a "vitaminoid *condition.*" It is the same change of viewpoint experienced by colloid science, which changed from Graham's colloid substance to the colloidal condition of Wo. Ostwald and P. P. v. Weimarn.**

The author first used the term "vitaminoid condition" in 1924, in a lecture delivered at the Innsbruck meeting of the Gesellschaft Deutscher Naturforscher und Ärtze. Afterward he learned that Kopaczewski had previously expressed a similar idea. He writes: "It seems to us that in the vitamin question, which has hitherto been approached by chemists with chemical concepts, colloid science might offer decisive elucidations."

The problem now reads: What physico-chemical condition is common to foods known to contain vitamins and to substances functioning as vitamin substitutes? After long investigations we found that the physical chemical property sought for was *surface activity.**

We next examined the surface activity of foods and found this hypothesis remarkably well substantiated. Before we started this investigation we thought the experimental part of the research would be short. Surface active sub-

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* These experiments show that the vitamin develops at the same time as *greening,* i.e. simultaneously with chlorophyll. *J. A.*

** Thomas Graham was thoroughly familiar with the fact that *the colloidal condition* is the important factor, and that the same substance, e.g., silica, could exist either in colloidal or crystalloidal condition. Graham's original classical papers should be read more often in the original, instead of depending on second or third hand deductions, erroneously drawn from them. See Index, Vol. I of this series. *J. A.*

* Several important papers dealing with surface tension are to be found in Vol. I of this series, especially those of W. D. Harkins, W. B. Hardy, I. Langmuir, and P. Lecomte du Noüy. *J. A.*

stances are those which lower the surface tension of water; it is more convenient to work with the positive value of surface activity than with the negative figures of surface tension. As the unit of surface activity we introduced the term *"Graham"* (abbreviated Gh), in memory of the founder of colloid chemistry. 1Gh. is, therefore, the force which lowers the surface tension of a solvent 1 per cent. The numerical value of surface activity is calculated from the formula $Gh = (1 - \frac{J_w}{J_x}) . 100$; where J_w is the surface tension of a solvent measured in a definite way and expressed in any units, and J_x is the surface tension of a solution of the unknown substance in the same solvent, expressed in the same units and determined by the same method. The stalagmometric method was used, for it gives values sufficiently accurate for our purpose, and its simplicity makes it suitable for a series of measurements. Table I shows the figures obtained. In measuring fruits, etc., the juices were clarified by centrifugation, and the aqueous extracts of flours, etc., were concentrated as much as possible, so that a fluid was obtained which just formed drops.

The data of the original report must be consulted for details of the preliminary treatment referred to in column 3; columns 4 and 5 show maximum and minimum values for surface activity, which were measured in preparations of different origin; as in all biological constants, the extent of variation is significant. Column 6 shows how many specimens of the same kind were measured. Column 7 gives the average surface activity from all measurements. Columns 8 and 9 show the content of vitamin B and C separately, according to Funk's excellent classification.

The table shows that in most cases surface activity runs parallel to vitamin content. Six substances, however, show irregular behavior, and an explanation of these "exceptions" at first offered considerable difficulty; finally a solution was found which not only explained the results, but formed a significant support for the whole theory. We investigated whether the surface active part of the juices and extracts was colloidally or molecularly dispersed. This was determined by an ultra-filter, a collodion membrane prepared by the method of Wo. Ostwald. The following interesting fact developed from this. In almost all substances the surface activity decreases only slightly after ultrafiltration; the active fraction is, therefore, molecularly dispersed. The surface activity decreased strongly in only four substances; i.e., in onion juice from 26.9 to 10.3; raw banana juice from 26.4 to 8.8, in the cooked from 23.3 to 6.5; milk from 32.0 to 8.7; and egg yolk from 29.0 to 7.3. These four substances belong to the previously mentioned "exceptions"; however, the surface activity of the ultrafiltrate corresponds exactly to the "vitamin" content of Funk's table!

Concerning the additional substances classed as exceptions, beer and coffee, there is the following to be said. The reason that beer produces no results in animal experiments may be attributed to the fact that the liquid has no natural connection with the diet, it works rather as a "poison" than as a "vitamin," especially if one tries to estimate the potency of the "vitamin" only from the weight curves of growing animals. We have seen significant prolongation of life by giving coffee, as well as isolated caffeine, to mice and rats which were fed a vitamin-free ration; this will be reported elsewhere.

If now the food and liquids are arranged according to their actual surface activity, i.e., the value for the ultrafiltrate set down for the corresponding

TABLE I.

No.	Substance	Treatment	Surface Activity		No. of Tests	Average Surface Activity	Vitamin Content	
			Maxim.	Minim.			B	C
1	Oats	whole grain	19.4	15.0	2	17.2	++	....
2	Rye	bread	14.1	12.2	4	13.2	++	....
3	Barley	whole grain	12.6	12.0	4	12.2	++	....
4	"	germ	7.9	7.5	4	7.7	....	++
5	Wheat	flour	8.0	4.2	3	5.5	(+)	....
6	"	germ	19.0	17.4	4	18.5	+++	0
7	Rice	polished	0.0	0.0	5	0.0	0	....
8	Maize	whole grain	15.0	13.9	3	14.3	+++	....
9	"	germ	19.4	21.0	4	20.0	++++	....
10	"	flour	0.0	0.0	3	0.0	+	....
11	Peas	whole grain	18.2	18.0	2	18.1	+++	....
12	"	germ	11.5	11.0	4	11.1	....	++
13	Beans	germ	11.9	11.6	2	11.8	....	++
14	White cabbage	raw	19.4	15.7	3	17.6	+	+++
15	" "	cooked	19.6	15.6	3	17.5	++	+
16	" "	slowly dried	11.0	9.6	3	10.3	++	0
17	Potato	raw	28.0	(16.3)	6	24.3	....	+++
18	"	cooked	23.0	22.5	3	22.8	++	++
19	"	slowly dried	14.8	14.4	2	14.6	(++)	0
20	"	steamed	9.9	8.9	3	9.2	....	+
21	"	peel	15.6	15.6	2	15.6	++	0
22	Red cabbage	raw juice	13.6	12.5	3	12.9	++	0
23	Tomato	raw	12.9	15.3	4	13.9	++	+++
24	"	heated	10.0	11.5	3	10.7	....	++
25	"	dried	11.8	11.4	2	11.6	....	++
26	Onion	raw	16.9	26.2	4	26.5	++	(++)
27	Spinach	raw	27.3	24.9	3	26.5	++	++++
28	Orange	juice raw	18.0	13.6	5	15.6	++	++++
29	"	" cooked	12.5	10.0	3	11.2	....	+++
30	"	" dried	12.7	12.1	2	12.4	++	....
31	"	" after adsorption	9.0	6.8	3	7.2	....	+
32	"	peel	14.6	14.0	3	14.3	++	++
33	Citron	raw	8.5	9.4	3	9.0	....	+
34	Lemon	"	22.9	22.2	5	22.6	++	++++
35	Grape	juice	6.3	6.3	2	6.3	?	+
36	Apple	raw	7.3	5.5	4	6.4	+	+
37	Pear	"	6.4	6.1	4	6.2	+	....
38	Banana	"	27.9	23.7	3	25.8	+	+
39	"	cooked	23.3	...	1	23.3	....	0
40	Beer	bright Pilsner	33.0	29.8	3	31.2	+?	0
41	"	dark Münchener	38.2	38.0	2	38.1	+?	0
42	Coffee	roasted	20.8	...	1	20.8	+	....
43	Yeast	autolysate	26.4	24.8	4	25.3	++++	0
44	"	heated	23.0	22.2	3	22.5	+++	....
45	Milk	raw cow's	32.9	31.0	4	32.3	+	+
46	Butter	raw	1.8	0.4	4	1.4	0	....
47	Egg yolk	"	29.9	29.9	2	29.9	++	....
48	Liver	raw ox	21.0	20.4	3	20.6	+++	+
49	Heart	" pig's	17.0	16.6	3	16.9	++	....
50	Fish	" cod	2.0	1.5	3	1.8	+	....
51	Honey	see text	8.4	4.8	2	6.6	....	0

substance, a series is obtained as shown numerically in Table II. If the data are expressed graphically, we lay off as abscissas the surface activity, and as ordinates the per cent of substances examined of a vitamin class which show the corresponding surface activity. Thus we have "distribution curves." From this grouping we find that foods whose extract or juice shows a surface activity of 0 to 1.4 Gh. are to be considered as vitamin-free in the old sense; substances which have values of 1.5 to 9 Gh. have slight vitamin content; substances with surface activity 10 to 17 have a moderately strong vitamin content; finally those foods known to have a high vitamin content show surface activity of 17 to 26 Gh.

Particularly interesting is the change in vitamin content, and parallel thereto in surface activity, on physical treatment of one and the same food. Thus the vitamin content of potatoes ($+ + +$) decreases on cooking and slow drying ($+ +$), and becomes quite weak on steaming ($+$); correspondingly the surface activity decreases from 24.3 to 14.6, and then to 9.2 Gh. Orange juice shows similar behavior; its 4 $+$ vitamin content in raw condition falls on drying to $+ +$, and after adsorption with kieselgur becomes $+$; the corresponding values for surface activity are 15.6, 13.4, and 7.2 Gh. Yeast does not deteriorate much on heating, but still noticeably; the surface activity drops only from 25.3 to 22.5 Gh.

How does the above tabulated expression of vitamin action in terms of surface action, apply to the somewhat limited results from a pure preparation of "the vitamin"? In the pursuit of our working hypothesis, this point was developed: to look for that constituent of the food which gives to it its surface activity. As we have not the means for analyzing according to American measurements, we have been able to develop the theory only on a modest scale, after the example Funk gave, wherein we work with a hundredth part of the Funk quantum. According to Funk's data, if dried yeast is extracted with alcohol and the sulfuric acid extract of the residue triturated with acetone, 1 g. of insoluble material is obtained from 1 kg. of yeast. This fraction should contain all the vitamin. The surface activity of the original material was 25.9 Gh., that of this fraction 25.2 Gh.

Because of the small quantity of material, we carried out only the next step in the isolation (decomposition with neutral lead acetate and precipitation with picric acid); the "vitamin containing" picric acid filtrate gave 24.8 Gh., the "vitamin-free" fractions from acetone extraction and picric acid precipitation, however, showed 4.2 and 2.0 Gh. It would be of great interest if our professional colleagues who are engaged in isolating the vitamin, would direct their attention to the agreement which we have reported between the so-called vitamin content and surface activity.

Our new theory of vitamin action developed from the original working hypothesis is as follows: *The so-called vitamin-containing substances act not by means of a chemical constituent in them, but by virtue of their inherent surface activity.*

Our problem now was the substitution for an unknown thing, the vitamin, surface activity, whose action was likewise unknown in the sense of a vitamin reaction.

The key which we sought for understanding surface activity as a reaction of the vitamins, was given in an interesting report by Brinkman and v. Szent-György, which concerned the permeability of membranes under the

TABLE 11.

Surface Activity	Vitamin Content			
	None	Weak	Moderate	Strong
0.0– 1.5	Rice, polished. 0.0 Butter, raw ... 1.4	Maize, flour .. 0.0		
1.5– 3.0		Fish 1.8		
3.0– 4.5				
4.5– 6.0		Wheat flour... 5.5		
6.0– 7.5		Pear .... 6.2 Grape wine ... 6.3 Apple .... 6.4 Honey .... 6.6 Orange after adsorption .. 7.2	Egg yolk 7.3	
7.5– 9.0		Bananas ...... 8.3 Milk .... 8.7 Citrons 9.0	Barley germ .. 7.7	
9.0–10.5		Potatoes, steamed 9.2	White cab.....10.3 Onions 10.3	
10.5–12.0			Tomato, heated .....10.7 Pea germ 11.1	Orange, cooked 11.2
12.0–13.5			Tomato, dried ......11.6 Bean germ....11.8 Barley, whole grain.12.2 Orange, dried.12.4 Rye bread 13.2	
13.5–15.0			Rutabaga ....13.6 Orange peel...14.3	Tomatoes ....13.9 Maize, whole grain 14.3
15.0–16.5			Potato, dried..14.6 Potato peel ...15.6 Heart 16.2	Orange 15.6
16.5–18.0			Oats, whole grain ....17.2 Wh. cabbage, cooked 17.5	Wh. cabbage..17.6
18.0–19.5				Peas, whole grain ......18.1 Beans, whole grain ......18.1 Wheat germ ..18.5
19.5–21.0			Liver *19.6	Maize germ...20.0
21.0–22.5				Yeast, heated..22.5
22.5–24.0				Lemons 22.6
24.0–25.5				Potato ....24.3 Yeast, auto-lysate 25.3
22.5–27.0				Spinach 26.5

* Original has 29.6.

influence of surface active substances.* The Dutch authors had found that the colloid coloring matter of blood, hemoglobin, which normally is held back by a collodion ultrafilter, passes undecomposed through the membrane if the latter is treated previously with solutions of surface active substances. Traces of these substances suffice to keep the filter permeable to hemoglobin for an almost unlimited time. The speed of diffusion is not changed by this treatment. On this basis the example, namely diuresis, which the authors themselves offer for their colloidobiological interpretation, scarcely serves as any explanation, as diuresis is characterized by increase in fluid volume and not by increased passage of colloids. How far other pathological conditions of permeability in the kidneys, for instance, diabetes, albuminuria, paroxysmal hemoglobinuria, etc., may be explained by the observed membrane effect, will be discussed in a later report of investigations already started.

We were concerned, therefore, in applying the example of Brinkman and v. Szent-Györy to conditions in the intestinal tract. Chyme passes through the intestine in which the foods are found as high sugars, as products of protein cleavage, amino acids, or dextrin, etc., therefore in a state of division which is characterized by low diffusibility; in addition surface active substances are found normally in chyme. Under their influence the colloidal, or molecularly dispersed food substances pass through the membrane of the intestinal wall into the blood stream. The avitaminoid condition is characterized by absence of surface activity in the chyme, so that, gradually, material necessary for the nourishment of the organism can no longer pass through in correctly balanced proportions; according to Tscherke, the absorption of nutriment is so poor with deficiency of vitamin B, that food acts like poison. A condition therefore develops which is like starvation, but not fully identical with it, since in vitamin deficiency, that is to say, with lack of surface active material, individual substances of smaller molecular weight always occur in the blood stream. The nature of these differs according to the diet with which the animal is fed; if it consists of polished rice and similar products, the substances which have diffused bring on a beri-beri condition; if other material is present the symptom complex of scurvy develops, etc.

As above stated, Brinkman and v. Szent-György show that a single treatment with the surface active substance made the membrane permeable to hemoglobin for an indefinite time. In application to intestinal processes this would indicate that a single "vitamin administration" would make forever possible the diffusion, the resorption of foods. But this is not so. It developed from further experiments which we performed, that duration of permeability in Brinkman's experiment is unlimited only if the diffusing material is itself surface active; it holds the way open itself, so to speak, when once it is in the pores of the membrane. As we have shown elsewhere, the membrane effect proceeds quite differently when surface active colloids are tested; in these cases the permeability is temporarily unlimited. The analogy with the behavior of vitamins is evident here. It may be inferred from a statement of Funk's that a certain amount of vitamin is to be considered sufficient for only a certain nutritional quantum.

In closing we must submit proof that actual surface active substances can serve as substitutes for vitamins. If we are concerned with the clinically

* R. Zsigmondy and F. N. Schulz found that in the presence of protective colloids, colloidal gold would pass through membranes otherwise impermeable to it. Moistening intestine with bile induces permeability to certain substances. See "Colloids and the Ultramicroscope," by R. Zsigmondy, Chapter 14 ("Zur Erkenntnis der Kolloide"); also J. Alexander, *Science*, 65, 207 (1927). *J. A.*

important aspects, as in the vitamin question, observations at the sick-bed offer evidence of great weight, far more than all the animal tests and test-tube experiments. For this reason two histories are offered—taken from an abundance of similar observations made by us—which show particularly well the substitution action in humans of surface active substances (coffee and alcohol) in place of vitamins. Both cases came under observation in the Eppendorf Hospital at Hamburg, and we thank the medical director, Prof. Dr. Brauer, for permission to publish them.

The first case is that of a 34-year-old shoemaker, who, for about three and one-half years, had been nourished exclusively on bread, meat, and conserves. During this time, he had had no illness and had not lost weight. Suddenly he awoke one morning with a severe stomatitis and greatly dilated blood vessels in the subcutaneous connective tissue of the extremities. At first there appeared to be no etiologic moment for the outbreak of the scurvy. After exhaustive questioning the following interesting history was obtained. Three days before the patient's illness, his landlady was taken sick and removed to the hospital; the only resultant change in the patient's manner of living was that he was no longer able to obtain his usual morning coffee. According to his statement, he drank two to three cups of strong bean coffee early every day. We do not assert that the strongly active coffee had supplied the surface activity necessary for unlimited resorption during the three and one-half years of a diet without vitamin, i.e., surface active foods. This substitution could have been sufficient only in an emergency. In other words, the malcompensation of the resorption disturbed by faulty nutrition, was balanced by the coffee; the condition must therefore afterward have become evident immediately, when this last saving agent for resorption failed.

The second case is that of a 53-year-old official, a heavy drinker, who had lived for twenty years "vitamin-free," that is, almost exclusively on bread and meat. After nine years he developed scurvy, for which he was treated in a hospital; after a dietic cure he was discharged as healed. He then followed his usual diet, which was made up almost exclusively of bread and meat. He continued well during the tenth year. According to the patient's statement, as well as to the supplementary information which the health official, Dr. Keck, most kindly placed at our disposal, the alcohol consumption of the patient was very considerable. In addition to numerous glasses of grog, his "night-caps" consisted of ten to fifteen glasses of beer, and a like number of kümmel. In the summer of 1925 the patient had a light grippe, because of which he had to go to bed for several days; the decreased drinking which resulted brought on hemorrhages. He did not, however, go to the hospital, in order "not to spoil his leave." During this attack his consumption of alcohol increased, with the result that although the diet had not been improved by addition of the so-called "vitamins," the symptoms disappeared completely. On December 15, 1925, the patient became sick with bronchitis; he had to remain in bed, but tried to satisfy his alcohol craving by liberal amounts of cognac. When the attending physician, at the request of the patient's wife, forbade the alcohol, and this prohibition was thoroughly enforced by the woman, five days later severe hemorrhages appeared in the upper extremities, on account of which he was taken to the attending physician at the Eppendorf Hospital. This case is particularly interesting because five days before the outbreak of scurvy, the doctor's examination, which on account of the bronchitis, in-

cluded an exhaustive inspection of the oral cavity, had shown no indication of scurvy.

These two examples may suffice; they show that surface active substances can control the onset of scurvy. In most cases of scurvy and beri-beri, the diet of the patient gives the etiology of the disease, but one seldom finds a clue to why the avitaminosis develops just at the time when the first manifestations are actually visible.

Animal experiments demonstrated further that with surface active substances of any kind, one could improve the weight curves of rats and pigeons which were fed on a vitamin-free ration, that is, with a mixed ration that was not surface active; (for instance, the mixtures described by Funk, by Mc-Cullom and Simmonds, contain no molecularly dispersed surface active material). That one never can obtain a pure control curve with these substances is due to the fact that all synthetic surface active substances exert a pronounced poisoning on the organism. The only harmless substances of this kind are vegetable extracts and fruit juices, which, for obvious reasons, could not be used. For the details concerned, the original work should be consulted.

In another series of experiments we were able to show that the surface activity of the chyme was decreased strongly if the diet was vitamin free. It is indeed a logical objection to our theory that in the stomach and upper part of the intestinal tract, the food is so changed that the surface activity which was measured before the consumption of food, is no longer accurate for the processes in the small intestine. Actually the values run parallel, since the bile secretions subside and a vicious circle is thus established.

Other experiments for the strengthening or weakening of our theory were carried out. Loops of intestine which were isolated *in vivo*, were filled with solutions of sugar or albumen, with and without surface active substances, and tested to see whether the concentration of the solute decreased greatly if surface active material was added. Much work has been published on the effect of these substances on resorption, but all lay emphasis on resorption *volume*, not on resorption *concentration*. On account of the secretions continually to be expected in the alimentary canal, the data of these differ widely. Traube asserts the action of the substances; Török and Buglia could not determine any. It is clear from the above that not the volume of the solution, but the quantity of dissolved material which it resorbs, is of value to the organism. The experiments of Brinkman and A. v. Szent-György also show that the filtration volume is not affected. In fact we determined that on the addition of octyl alcohol, atropin sulfate, caffeine, nonylic acid, as well as soaps (quite independent of the pH of the solution), the albumen or sugar concentration after removal from the intestine had decreased more strongly as a result of resorption than without the addition. The exact values of these experiments will be published soon.

We have made investigations with lower organisms also, paramoecia, yeast cells, etc. For these forms we have passumed a vitamin requirement according to Funk. We found that well-known "vitamins" such as orange juice, yeast autolysate, etc., were as potent as surface active substances in very dilute solutions. Similar reactions are obtained with substances affecting osmotic pressure; it is logical to assume that the production of permeability by surface active substances leads to psuedo-osmotic effects; experiments with regard to this are in progress. Results similar to those obtained with protozoa have been obtained also with higher plants.

In summarizing, it can be said that clinical observations, as well as experimental research, have shown that our colloidobiological vitamin theory possesses a high degree of plausibility. This theory states that the so-called vitamin containing substances act, not by means of a chemically definable constituent in them, but probably by their inherent surface activity, which is inherent in molecularly dispersed material.

Colloids in Pharmacy '

By John Uri Lloyd, Cincinnati, Ohio

Restricting thought to the pharmacy of plant structures, one may allow that the study of systematic pharmacy is based upon colloidal aggregations and colloidal reactions. Consider the blanket terms—tinctures, syrups, wines, elixirs, infusions, decoctions, fluid and solid extracts. These and such as these apply to complex associations, mixtures, adhesion compounds, structureless materials and mass complications. They are "bunched" together by means of liquid solvents and liquid excluders in which the totality of it all is made up of colloids derived from the vegetable structures that contain or yield them.

Inasmuch as from the beginning of man's historical records, processes such as are above mentioned have been employed in medicine manipulation, it may be largely argued that the study of plant pharmacy has ever been and is yet based upon colloidal complications. And inasmuch as the normal structures and juices of most, if not all, plants are colloidal, it fundamentally follows that the study of pharmacy as applied to natural plant structures is that of colloidal research and colloidal manipulation.

Nearly a half century ago this writer became enthusiastic in his views concerning the attributes of plant structures lying in the borderland and even outside the limits of what was then included in both legitimate chemistry and recognized pharmacy. In his lectures and contributions to print he usually employed the terms *structural affinity* and *mass actions* to express colloidal reactions and attributes outside the realms of established atomic and molecular chemistry. This field of investigation from his viewpoint, proved a wonderland of mysteries that become more entrancing as the years passed, and yet increasingly perplexing. The "irregularity" of concept approached the fanciful, as pharmacy was then taught, and with well meaning advisers was handled with a degree of hostility based on the accepted views concerning atomic migrations in which colloidal compounds as well as adhesion phenomena were neglected. But within recent years such mental reasonings regarding extension possibilities are more generously received, partly because of the clearer view that now prevails regarding the utility of structureless compounds, partly because advanced thought in pharmacy more fully comprehends that its inherited opportunity, voiced in the pseudo-pharmaceutical compounds of times gone by, is really scientific in both theory and application.

But colloidal pharmacy is not now and has never been restricted altogether in its application to plant and animal. Possibly the nearest approach to semi-official recognition of the colloid in pharmacy was *Dialyzed Iron,* a preparation in which most of the crystallized chlorides or sulfates are, by means of bladder or parchment dialyzers, separated from the colloidal iron

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* In this attempt to abridge a subject that has taken the writer's life work, it must be comprehended that details essential to the problem as a whole are necessarily excluded. These pages give but a touch to the subject embraced in the title. *J. U. L.*

oxides. Even there the term *colloid* seems to have been, and yet is, totally ignored in pharmaceutical literature, the commercial as well as the descriptive title of the product, *Dialyzed Iron,* being yet universally applied.

In like manner, scaled ammonia-citrate of iron and potassium, as well as scaled tartrate of iron and potassium, then considered and called "amorphous" were a half century ago great favorites. All these lay within colloidal boundaries, to which it may be added that solution of ammonio-citrate of bismuth (Liquor Bismuth) might likewise be given a setting as the result of early pharmaceutical colloidal effort. Such as these answered in some respects the nature of Graham's colloids (although not made by dialysis). Surely the pharmacist need not go out of his home to find among the inorganics, very early colloidal recognition.

But pharmaceutical effort amid colloidal structures, although long dormant in its original home, has been recently and elsewhere very active. Renewed colloidal research in pharmacy came recently in the study of animal juices and products, all of them structureless, as far as this writer is aware, if molecular formulas capable of blackboard exposition be taken as the standard. Here, too, we find the term *colloidal* neglected, although products such as these wedge into the society of Graham's typical colloid, glue, which gave the very name "colloid."

In closely allied lines we find the term *vaccine* which seems expressive of a use of the product, and the term *serum* which reminds one of the juice, the makers of these products crystallizing none of them, even those most energetic. Let us now consider the vegetable field, in which, as already stated, colloidal problems have confronted the pharmacist since the dawn of history.

Very complicated is every natural plant structure, both as concerns its liquid and solid content. Interlaced and interlacing are the juices and the cells containing juices, that make the thing a living whole. In connection with that structure, we find that if it be normal, colloids, liquid and solid, dominate wherever research is applied. As the living animal is a colloidal collection, so is the plant that nourishes the animal. Let us now briefly consider in the vegetable field some of the problems confronting the pharmacist.

Accepting that interlaced, non-crystalline substances compose the major part of either fresh or dried plant textures, it becomes the pharmacist's duty in the object of obtaining a natural separate, to untangle these complicated structures without integral alterations that involve fundamental re-arrangements. In this object, the writer is convinced that heroic chemicals must be excluded from such manipulative processes. Even the influence of acid or alkali may be fatal to the equilibrium of structures as delicately balanced as are many plant colloids in their natural settings. Very kind must be the touch that to sensitively balanced plant structures, brings not textural reconstructions. For example:

In the process of extraction if natural colloidal groups be in mind, be it by means of maceration or percolation, the most promising neutral liquids in the writer's experimentation are embraced in the discriminative service of a set of fourteen that, several decades ago, were by him selected for elective solvent as well as excluding purposes. These are glycerin, water, alcohol (U.S.P. 1880) methanol, acetone, chloroform, amyl alcohol, acetic ether, sulfuric ether (U.S.P. 1880), benzone, carbon disulfide, benzine, turpentine oil

(rectified), and liquid petrolatum. (See *Proc. Am. Pharm. Assoc.,* 1879-1884-1918, "Precipitates in Fluid Extracts," etc.) The chief components of the vegetable groups to be considered as making up bundles,* of which the constituents have friendly solvent attributes, are:

1. Materials soluble in water (gums, albuminates, glucose, sugar, earthy salt, compounds, many alkaloidal colloids, etc.).
2. Materials soluble in alcohol (resins, essential oils, flavone derivatives, some fixed oils and fats, chlorophyl, chlorophyl-wax, etc.).
3. Materials soluble in ether (fats, fixed oils, wax, etc.).
4. Materials soluble in glycerin (tannates and related bodies).

By means of the neutral solvents named (some substances are quite soluble in several of the liquids) successively applied, most plants, be they green or dry, may be practically abstracted of their distinguishing colloidal contents. Each saturate is, however, not an ultimate, but in itself may constitute a bundle of associated constituents which by further subdivision is capable of yielding yet more closely related sub-bundles.

Each primary bundle, regardless of the solvent used, may carry contaminations (adhesions) of the others. For example, if water be first used as a manipulative solvent, the product may be contaminated by adhering fat and resin, although when pure fats, resins and fixed oils are (unless in dispersed condition) practically insoluble in water. When undesirable in the final product, such a fat must be separated by an elective solvent. In like manner primary "bundles" derived by means of other solvents, may carry adhering materials whose location as regards solvents is elsewhere, and which may be transferred to their natural homes by subsequent manipulations with appropriate cleansing solvents. Transference of yet other adhesion slivers can be accomplished by means of yet other neutral liquids having the power of making closer cleavages.

In some cases the process of discriminative precipitation by means of a liquid affiliating with the original menstruum, but in which one or more of the constituents soluble in the original liquid is insoluble, may be employed to dissect a bundle of plant colloids. Thus, if the gum of an extracted drug is desired, the addition of alcohol to the water-extracted colloidal complexity makes a precipitate of the gummy part of the substance, practically free from water-soluble materials. Without the use of energetic chemicals, colloidal plant structures by such manipulative processes may be dissected and individualized to a degree of pharmaceutical satisfaction, even though not always to absolute chemical perfection. Occasionally unexpected destruction of natural structures follows the use of liquids seemingly innocent. For example, the pronounced alkaloidal structure in fresh Sanguinaria is utterly destroyed by contact with acetone for a very limited period. Chloroform likewise reacts destructively in special cases.

For special purposes dialysis may be employed. Spontaneous evaporation through an absorbent mass-medium is likewise often applicable, where capillarity differentiates colloidal mixtures closely connected as far as solvency is concerned. This particularly applies to separation of crystalline contaminations and to separation of coloring materials, where the drying of a magma in a muslin strainer is all-sufficient. The most soluble material passes

* The writer has long used the term "bundle" in this sense, but so far as he is aware, it is not elsewhere thus employed.

to the outside of the strainer. Purification by adhesion attractions, through a capillary medium such as blotting paper (paper pulp), may be absolutely necessary as a final separator of certain closely locked structures. This latter process enables one to make very complete cleavage separations of materials which bear nearly exact solubilities.

Whoever enters this field must needs liberate himself from some of the limitations concerning both thought and action embraced in past authoritative pharmaceutical processes as applied to plant structures. It should be comprehended that every root, bark, flower or herb is a separate creation, carrying group entities ("bundles") that need be individually studied in a new light as concerns applied pharmacy.

SUMMARY. Does not the fact that colloidal substances constitute the bulk of the animal and vegetable kingdoms; that colloidal phenomena dominate life-building as well as life-destroying processes in both; that the most valuable and kindly of all plant remedial agents, as well as those most energetic (does not this apply also to animal vaccines and serums?) are colloidal, bid one absorbed in seemingly distant fields, be patient with a neighbor entangled in the intricacies of these stupendous outreaches? Do not numberless examples in which crystallization is necessary to the purity perfection, of the material that assumes the crystal form, likewise bid him involved in the magnitude of the colloidal world of vegetation be very patient with the man content by reason of inherited views to argue that only structures that will crystallize are entitled to scientific recognition?

Lastly. In the rivalries of theoretical or academic discussion, the practical pharmacist has, as a rule, little concern, unless it be as an avocational diversion. His first aim is the utilization of materials serviceable to humanity—in this object he both thankfully accepts and gladly utilizes whatever comes from all directions. To him a jelly or a crystal alike appeals, if it be adapted to the relief of mankind's ailments.

The Significance of Colloid Chemistry for Pharmacology and Therapeutics

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I

In order to determine the therapeutic activity of a remedy and its mode of action, it is necessary to know three factors: (1) the action of these substances on the different cells and tissues; (2) the order in which the substance, in the manner in which it is applied, acts on the different organs and tissues of an animal, and (3) the possibilities of variations in these actions by pathological changes in the various organs.

1. The action of a substance on a cell or a tissue can primarily be a twofold one. First, upon its vitality, that is, upon the processes which are common to all cells as living substances (reproduction, etc.). This can best be tested on the power of reproduction of unicellular organisms, bacteria or protozoa. For example, if one places protoza which have been cultivated in hay infusion, in solutions of K_2HPO_4 for a short time, their rate of division will be accelerated for many generations afterwards. On the other hand, the addition of other substances can diminish their rate of division (*see, e.g.,* Calkins [1]). These would be effects upon the general functions of the cell.

Besides this there are other alterations of function which are characteristic for the individual species as, for example, changes in the contractibility of the muscle, changes in the contraction-volume of the heart, etc. These functions also can be altered qualitatively and quantitatively by poisons.

2. However, the knowledge of the mode of action of a substance tells little or nothing regarding its value in therapeutics. For this purpose, the order in which its various actions unfold themselves upon the different organs is most important. For example, not every substance which paralyzes the nervous system can be used by surgeons as an anesthetic. Such a substance must first paralyze the centers of the cerebrum, then those of the spinal cord, and only after this, the centers in the medulla oblongata. A local anesthetic, in the concentrations at which it acts as an anesthetic, must not injure the tissues. To a certain degree we can exclude some of these accessory effects by the manner in which the substance is used.

Another criterion for the determination of the therapeutic action of a drug is its indirect action. For example, we know that certain cells produce substances which either stimulate or inhibit the development of others; we know also that certain substances injected into an animal cause an increased secretion of adrenalin, and thus cause, indirectly, an adrenalin action.

With regard to the first question, the reader is referred to the investigations of Carrel,[2] Ber,[3] Haberlandt,[4] von Gaza,[5] Handovsky, du Bois and Krantz.[6] In this category belong also the older researches of Pasteur and the newer ones of Robertson,[7] that pure cultures grow much more slowly than those in which two or more individuals are present. Handovsky [8] has called such cell systems "heterovital"; it should be noted that not only in such cultures are cells of different vitality present, but also that the vitality of each individual cell is dependent upon the others, just as the heterodispersion of a colloidal system is necessary for the preservation of the state of the system as a whole.

Concerning the second question, Cannon,[9] Laidlaw,[10] Rogoff [11] and Eichholtz,[12] have called attention to the increased adrenalin excretion caused by nicotine and nicotine-like substances. Moreover it should be recalled that according to the investigations of Wieland [13] many poisons (picrotoxin, lobelin, camphor, etc.) act in such a way as to increase the sensitiveness of centers for the normal CO_2 stimulus.

For the investigation of therapeutic usefulness of a drug, it is very important to know not only the properties of a substance, e.g., its toxicity, but also the various properties of the very cells and tissues, on which the action is to be exerted, properties which we shall group under the conception of susceptibility to poisoning.[14] This is dependent upon many conditions. In the first place, different species of animals are not equally sensitive to the same substances. The porcupine is resistant to cantharidin and snake venom; the chicken is resistant to cantharidin, strychnine, arsenious acid; the dog can withstand several grams of morphine, while a few decigrams are fatal to much heavier human beings. Rabbits, guinea pigs, rats and pigeons are very sensitive to atropine. Besides these congenital immunities, there is an acquired immunity to various substances, as we know from serology. And also there is a congenital and an acquired hypersusceptibility to various substances, both among different animal species and in different individuals. We also recognize hypersusceptibility in some human beings toward iodoform, antipyrine, morphine, quinine, calomel, etc., i.e., idiosyncrasies about whose causal mechanisms we know little, and about which we can only say that they are occasionally observed. We have a somewhat better knowledge of the mechanism of anaphylaxis of hypersusceptibility since the investigations of Dale,[15] the hypersusceptibility which follows the injection of traces of foreign protein substances.* Dale has shown that not only the whole organism, but also separate organs (uterus, intestines, etc.) of the guinea pig which has been previously treated by an antigen, are hypersusceptible to this antigen even when they have been excised from the body and are studied in vitro. We have here, therefore, only a sensitization, that is, an alteration of the state of the body cells themselves.

[2] Carell, J. Exp. Med., numerous publications.
[3] Ber. Münch. Med. Wochschr., 1006 (1923).
[4] Haberlandt, Beitr. Allgem. Botan., beginning with Vol. 2.
[5] Gaza, Arch. klin. Chir. (1922).
[6] Handovsky, Du Bois and Strantz, Arch. exp. Path. Pharm., 100 (1924).
[7] Robertson, Biochem. J., 15 (1921).
[8] Handovsky, "Grundbegriffe der Kolloidchemie," Berlin, 1923.
[9] Cannon, J. Pharmacol., 3 (1911).
[10] Dale and Laidlaw, J. Physiol., 45 (1913).
[11] Stewart and Rogoff, J. Pharmacol., 13 (1919).
[12] Eichholtz, Arch. exp. Path. Pharm., 99 (1923).
[13] Wieland, Arch. exp. Path. Pharm., 95 (1922).
[14] Handovsky, Kolloid Z., 30 (1922).
[15] Dale, J. Pharmacol., 4 (1913).
* See also paper by A. Lumière in this volume. J. A.

Eppinger and Hess [16] have shown further that persons with high vagus tone are particularly sensitive to pilocarpine which normally stimulates the peripheral vagus endings, whereas persons with increased tonus of the sympathetic nerves react strongly to adrenalin which stimulates the sympathetic endings. We are particularly indebted to the investigations of Burridge,[17] Pick, Amsler, and Kolm,[18] for doing much to clear up the obscurities in this field. In these experiments, the isolated frog's heart was made artificially "vagotonic" by previous treatment with acetylcholin, which stimulates the vagus endings and thus increases the vagus tone of the heart. They have also treated the heart with ergotoxin which paralyzes the sympathetic endings and in this way gives rise to a relative over-action of the vagus. On such hearts, even adrenalin, which is ordinarily exclusively sympathicotropic, acquires a vagotropic action, that is, it gives rise to diastolic cessation of the heart beat which can be prevented or removed by atropine. Likewise they were able to make the heart artificially sympathicotropic so that muscarin or acetylcholin, which are normally vagotropic, acquired a sympathicotropic action. The tonus of the muscles or the degree of stimulation of the nervous centers is an index of their sensitiveness to poisons. Certain pathological operations can change the sensitiveness of the tissues to certain poisons. According to Klemensiewitz, adrenalin has almost no action on the blood vessels of inflamed tissue, and Macht and Giu Ching Thing [19] have observed that the bronchial muscles of hogs affected with pneumonia are insensitive to a series of, poisons (pilocarpin, muscarin) which have a definite action upon the same organisms of healthy animals.

As we see, it is therefore possible to discover definite relations between the *physiological condition* of an organ and its *sensitiveness* to individual drugs, but the physiological and pathological conditions which determine this are still very complicated phenomena.

Pathological anatomy and pathological chemistry enable us to recognize the gross macroscopic or microscopic operations of the living cells. However, these are not the things which the physician usually has to modify by treatment, but more frequently, functional disturbances which are neither morphological nor chemical.

However, where our morphological or chemical methods are ineffectual, we may look for aid to colloid chemistry, which explains, among other things, the relation between form and chemism. We know these relations from swelling phenomena : a cube of gelatin is able to alter its form and volume by the taking up and giving off of water through small alterations in the ion content of its environment. We know the conditions governing this reaction, e.g., of acids with the amino groups of the gelatin, and we know the dependence of these changes of form upon these chemical reactions.

Protoplasm is also such a colloid system. We have called it a mixed colloid [20] in order to give expression to the fact that it is a single system which contains chemically different substances which must be in a definite equilibrium somewhat comparable with the colloid chemical system in a case of protective action, where we also have an equilibrium between the protected colloid, the protecting colloid and the electrolytes and water which surround them.*

[16] Eppinger and Hess, "Die Vagotonie," Berlin, 1920.
[17] Burridge, *J. Physiol.*, 41 (1911).
[18] Pick, E. P., Amsler, C. and Kolm, R., *Arch. exp. Path. Pharm.*, 84 (1918).
[19] Macht and Giu Ching Thing, *J. Pharmacol.*, 18 (1921).
[20] Handovsky, "Leitfaden der Kolloidchemie fuer Biologen und Mediziner," Dresden, 1922.
* The cumulative protective effect should not be overlooked. See J. Alexander's paper on "Protection" in Vol. I, this series; also paper by D. T. MacDougal in this volume. *J. A.*

II.

The condition of the non-vital colloids and their alterations or changes of state are determined by surface tension, hydration and electric charge. We must therefore concern ourselves in the question of whether these forces are also of equal importance within the cells, for only then can we determine what rôle the colloidal state of the components of the cell play in the cell functions. Under the term "functions of the cells" we understand the sum of the processes by which they react to their environment under different conditions. Substances and energy are consumed and transformed, and through this the physiological state of the cell and usually also its colloids, are altered. The relation between these two changes in condition will be discussed later. The inter-actions of the cells with their environment must always be borne in mind in order to comprehend life processes. In order to facilitate the comprehension of these relations of the mutual inter-action of cells and environment, we must divide these into their individual processes which we have recognized as hydration, surface affinities and electrical charge, and we must therefore speak of the metabolism of water, of surface affinities, and of the electrical charge of the cells and tissues, and see if these properties are as important for the activity of cells as they are for the state of the individual colloids.

WATER METABOLISM

The great significance of the water metabolism of the cells and tissues, is manifested by the fact that no life is possible without water and that water plays a very important rôle in the individual isolated hydrophilic colloids of the cells.

Water metabolism depends, in the first place, upon the water content of the cells. This is, however, by no means a constant quantity either for the species of cells or for the individual cell, but on the other hand, its constancy increases as we ascend in the animal scale. The cells of the higher animals contain approximately 80 per cent of water; cells exhibiting the lowest metabolism, e.g., connective tissues and fatty tissues, are considerably poorer in water; the lower animals often contain much more. For example, the umbrella of the Medusa contains 95 per cent but nevertheless has a definite structure. Higher animals alter their water content within rather narrow limits; thus starving animals can lose all of their fat, 50 per cent of the protein, but only 10 per cent of their water. In lower animals a much greater adaptation to environment is possible. Frogs, at lower temperature where the metabolism is very slight, can lose 50 to 60 per cent of their water, so that their tissue instead of 80 per cent may contain only 22 per cent of water.[21] This adaptability in regard to water is much greater with fungi and bacteria.

In the individual animal, the water content depends particularly on the age and the functional activity of the cells. As regards age, it is well known that cells in individuals lose water as age increases, so that, for example, the newborn child contains 66 per cent water and the adult only 58 per cent.

Besides species and age, the water content of cells is also greatly dependent upon the cellular activity. Even in yeast cells it is known that swelling takes place, that is, water is taken up, during fermentation. In higher animals it is

[21] Durig, *Arch. ges. Physiol.*

also found that the water content increases in activity as compared to rest. Thus it has been shown[22] that the venous blood which emerges from the salivary glands becomes concentrated when the gland has been stimulated and that the cells of the gland take up water in proportion to the amount of saliva excreted.

For a given average physiological condition we assume a mean water content for each kind of cell, and we may ask, how does an alteration of this mean water content, or in other words, a true water metabolism, take place? It can only occur by overcoming those forces which tend to maintain the mean water content at a constant level. These forces are, however, quantitatively and qualitatively different for different cells. Thus in the experiments upon the desiccation of frogs, it has been shown that muscle and skin give off water more readily, and the heart and the brain scarcely lose water at all, under conditions in which the animal loses 30 per cent of its body weight.[23]

What are the characteristics of the forces which hold the water in the cell? We must differentiate two distinct types: osmotic forces and the swelling of the colloid components of the cell. *Osmotic forces* can act only where semipermeable membranes are present, which are permeable for the solvent but impermeable for some or all of the dissolved substances. When cells are surrounded by such semi-permeable membranes and when they are placed in a hypotonic salt solution, that is, in one whose salt content is lower than that in the cell protoplasm, water must enter the cell and its volume must be increased. Such semi-permeability has been definitely demonstrated in many plant cells; in fact, it was on such plant cells that the first laws of osmotic pressure were demonstrated by Pfeffer and de Vries. It was on the mid-rib of the leaves of *Tradescantia dicolor* that Pfeffer undertook experiments on osmosis and demonstrated that a difference of concentration of 0.0025 mol exists between the cell fluid and the cell environment.[24] For these experiments only those cells are suited which have a very thin cellulose membrane, a thin tube of protoplasm and a large amount of space for the cellular fluid. Such cells offer the most favorable condition for osmotic interchange, but total pressure in the interior of the cell depends not only on the osmotic pressure, but also on the elasticity of the membrane, the surface tension which exists between the protoplast and the environment (central pressure), and finally, the imbibition of the swelling of the cell colloids. Experiments on the isolated organs demonstrate also that the *swelling* of the colloids of the cells play a rôle in their taking up and giving off of water. From this it follows that the imbibition of water from isotonic solution is by no means uniform, but that it is, on the contrary, much more marked in acids and alkalis, and that the increase of the water content brought about by acids and alkalis is inhibited by small quantities of salts; in other words, by the antagonistic action of salts, as we have learned through the effects of salts on the simpler proteins. Accordingly the weight of the gastrocnemius muscle of the frog which has been immersed for eighteen hours in salt solutions, increases 50 per cent in $N/10$ KCl, 5 per cent in $N/8$ NaCl, 0 in $N/8$ LiCl, but on the other hand, decreases 20 per cent in $N/8$ CaCl$_2$ (Overton). This cannot be an osmotic phenomenon. The same uniformity is found in red blood corpuscles, lenses and entire eyes.[25] True

[22] Asher.
[23] Durig, *loc. cit.*
[24] Fitting, *Jahrb. wiss. Botan.*, 56 (1915); 57.
[25] Fischer, M. H., "Oedema," New York.

osmotic equilibria can be demonstrated with blood corpuscles.[26] We must accordingly assume both factors to be active in animal cells which in contrast to plant cells are entirely filled with protoplasm and have no absolutely demonstrable membranes; under certain circumstances these factors may be interrelated.[27] In any case, the establishment of a salt and water equilibrium, which is influenced by osmotic factors, becomes more difficult with increasing viscosity of the protoplasm; for the osmotic equilibrium is a consequence of molecular movements and the velocity of the latter depends upon the viscosity of the medium. The ions act only to charge or discharge the cell colloids, and thereby influence alterations in the water transport of the cell which we shall discuss in detail later. All these phenomena accordingly are dependent upon one another, and take part in the water transport of the cells. If we wish to differentiate systematically between the fundamental significance of the osmotic and colloid-chemical factors, we can say that the static maintenance of the water equilibrium is due to the osmotic factors, but the dynamic alterations is due to the factors which influence swelling and electro-osmosis.

The water equilibrium of the individual cell is largely determined by the salt content of the ambient fluid, particularly by the proportion between the concentration of alkali and bivalent ions, while the strikingly high sodium chloride content of the intercellular fluid of higher animals is of great importance for the osmotic equilibrium between the cells and their environment. There is, under normal conditions, a tendency to maintain the water content of the blood of higher animals at a constant level. If, for instance, a large quantity of hypotonic salt solution is injected into a vein of the dog, the major portion of the water is immediately transported to the muscles and causes there a decrease of osmotic pressure, which can be measured in the expressed juice, while the osmotic pressure of the blood remains unchanged. When, however, concentrated salt solutions are injected, both salt and water remain in the blood for a certain length of time.

Metabolic processes, especially the oxygen exchange, also play a rôle in the stabilization of the water content. An insufficient oxygen supply gives rise to the production of acids which cause swelling of cells, *i.e.*, an increase of water content or at least its more tenacious retention (Hofmeister, M. H. Fischer).

Not all tissues are to the same degree able to participate in the water regulation. The higher mammalian organism for instance, has two water depots: one in the muscles, which constitute about four-tenths of the body weight and contain about four-sixths of the depot water, and the other in the skin, containing about one-sixth of the depot water. There is also a relation between water content and activity, as was already previously stated. The irritability of muscles, for instance, is increased in hypotonic solutions. Furthermore it is well known that the irritability of dehydrated frog muscles is lower and that especially the latent period following indirect stimulation is considerably increased with the degree of dehydration (Durig, *loc. cit.*). It follows that osmotic forces have a dominant influence only on the metabolism of certain cells, mainly of those containing a relatively thin fluid, such as certain plant cells and perhaps also erythrocytes. In the majority of cases, however, water metabolism is determined or at least largely influenced by the properties of the cell colloids, their viscosity, swelling capacity, and electrical charge. The

[26] (Hamburger and) Takei, *Biochem. Z.*, 123 (1921).
[27] Ostwald, Wo., "Grundriss der Kolloidchemie."

action of one of these factors alone will manifest itself only in extreme cases. The combined action of these three factors may become more comprehensible by the assumption that there are two kinds of water in the cell: free water and swelling water.* Both come equally into consideration as solvents according to the experiments of Hofmeister.[28] There undoubtedly exist intermediate stages, and in each instance the water is bound with different and varying tenacity. We know, for instance, that the dehydration of a 10 per cent gelatin solution requires 700 atmospheres, while 1,250 atmospheres are necessary for the dehydration of a 25 per cent solution. *The varying ratio between free and bound water and the varying resistance to dehydration, which depends on the former, is the factor determining the importance of the water economy of cells and tissues for their functioning.*

Surface Forces in Cells and Tissues

Another group of properties which is of great importance for the metabolism of the cells, is their surface affinities. They are determined by the free surface of the colloid particles and their surface tension. Their action consists primarily in binding surface-active substances and thus reducing the surface tension. This process is called *adsorption*.

The question arises whether there exist intracellular phenomena which can be interpreted as surface phenomena. *Oxidation phenomena* are, according to Warburg,[29] a very important instance of this group. The cell consumes oxygen in varying amounts according to life conditions. There is also the respiration of the erythrocytes, which is carried on by their solid constituents. Oxygen is not only bound by the cells; it is used up for the oxidation of oxidizable substances. On shaking amino-acids (cystine, tyrosine) with erythrocytes, CO_2 and H_2SO_4 are formed among other products, which proves that a partial oxidation has taken place. Warburg succeeded in demonstrating that this reaction is a surface reaction. The following is an outline of Warburg's reasoning: It has been known since Faraday's time that the velocity of the reaction between hydrogen and oxygen is increased by the presence of solid substances, such as ground glass, quartz, carbon, etc., and that the increase is caused by the reacting gases being concentrated on the surface so as to produce a measurable reaction velocity. The acceleration of reactions by surface forces is known under the name of adsorption catalysis.

It was shown later that an increase of reaction velocity can be brought about not only in the case of gases reacting in contact with surfaces, but also by adsorption of reacting components from a solution. This was first demonstrated by Freundlich for the oxidative decomposition of oxalic acid. Another significant instance of this kind is the oxidation of the cell's combustible materials, *i.e.*, the amino-acids, by shaking them with blood charcoal, which, as was found by Warburg, must contain iron in order to be effective.* Cystine and tyrosine were oxidized in exactly the same way as in the experiment with erythrocytes. It could be demonstrated that in both cases the *quantity of amino acid oxidized increased with the quantity adsorbed.* This is considered proof that the oxidation of amino-acids effected by shaking their solution with erythrocytes is also a surface phenomenon. Another proof is fur-

* For a classification of the several ways in which water is held by soils, see Vol. III, paper by G. Bouyoucos. *J. A.*
[28] Hofmeister, *Arch. exp. Path. Pharm.*, 28, 217 (1891).
[29] Warburg, *Biochem. Z.*, beginning 119 (1921).
* Extremely minute amounts of iron suffice. See also paper by G. Bredig, in this volume. *J. A.*

nished by the following fact: The oxidation of amino-acids in contact with animal charcoal or cell surfaces can be inhibited by anesthetics. Since anesthetics diminish the adsorption of amino-acids from solutions, this inhibition of oxidation appears as an adsorption displacement. Consequently oxidation is a surface phenomenon, which according to Warburg takes place only in the parts of the surface containing the catalyst, iron.

The inhibiting action of anesthetics on respiration increases proportionately to their displacing capacity. The inhibiting effect of anesthetics therefore depends not on their concentration but on their adsorbability, as is demonstrated by the following table of Warburg. The table gives a number of anesthetics in concentrations in which they inhibit the oxidation of amino-acids on charcoal to 50 per cent. c is the concentration of the anesthetic, x the quantity adsorbed by 1 gram of animal charcoal.

<div align="center">TABLE I.</div>

	c	x
Dimethylurea	0.03	1.1
Diethylurea	0.002	0.68
Phenylurea	0.0002	0.76
Acetamide	0.17	1.2
Valeramide	0.003	0.62
Acetone	0.073	1.33
Methylphenylketone	0.0004	0.73
Amyl alcohol	0.0015	0.87
Acetonitrile	0.2	1.5

The concentrations required to produce the same effect vary in the proportion of 1 : 1000, while the ratio of the extreme adsorbability values is only 1 : 2.5. It is evident that only the adsorbability matters.

Surface reactions are of a far more general significance. Not only oxidations, but also all fermentative processes in the cells are to be considered as surface reactions. It has been known for a long time that colloidal metals in minute quantities are, like ferments, able to accelerate reactions. This analogy between the kinetics of ferments and inorganic catalysts is illustrated by the fact that the action of both can be inhibited by the same poisons.[30] Besides the anesthetics, heavy metals have this effect (see below); but even substances exerting a surface action only, are also capable of inhibiting ferment reactions. Saponin, for instance, inhibits the action of the soy bean urease,[31] which converts urea into ammonium carbonate. The inhibition is in this case the result of a direct decrease of surface tension.

Logically, any substance *which increases adsorption* should favor the binding of surface-active substances. This too has been experimentally confirmed. The hemolysis of red blood cells by saponin is, for a given medium, proportional to the saponin concentration. If, however, the erythrocytes are suspended in an isotonic sucrose solution, or if the isotonic condition is maintained by mixing sucrose solutions with increasing amounts of NaCl solution, then the hemolytic effect for a given saponin concentration is increased with the salt content.[32] An analogous phenomenon is the increase of acetic acid fixation by animal charcoal through the action of NaCl.[33]

[30] Bredig, "Anorganische Fermente," 1901.
[31] Bayliss, "The Nature of Enzyme Action," London.
[32] Handovsky, *Arch. ges. Physiol.*, 190 (1921).
[33] Wiegner, *Kolloid Z.*, 28 (1921).

These experiments establish with a high degree of probability the dependence of cell sensitiveness to poisons upon the colloidal state of the cells.

The foregoing part of this paper treats repeatedly of surface reactions in cells, adsorption compounds, adsorption displacements, increase of adsorption. Which surfaces are supposed to take part in these phenomena? The surfaces of the individual, primary or secondary particles of the colloidal cell constituents, or the outer surface of the cell? It is impossible to give an exact answer to this question. A change in the internal surfaces would result in a *different state of gelation,* which could be determined by the viscosity of the protoplasm. Consequently anesthetics in concentrations causing paralysis ought to be capable of increasing the viscosity of the protoplasm. This is actually the case. Heilbronn [34] heated cells from the starch sheaths of *Vicia Faba* to 25-35° C., without producing a change in their viscosity or geotropic excitability. When the heating was continued for one-half hour at 45° C. the viscosity was increased and the irritability reduced. The same observation could be made for the paralysis of the geotropic irritability in the same cells. The viscosity of protoplasm is increased in 0.4 to 1.6 per cent solutions of ether in water which reduce or destroy the geotropic irritability. In these cells a decrease of irritability is correlated with an increase of viscosity. This relation will be discussed later in connection with the paralysis of cells.

Other physiological processes also seem to be related to certain viscosities of the protoplasm. The gradation of protoplasm viscosity seems to be particularly fine in the various stages of cell division. L. V. Heilbrunn [35] * has shown on worm eggs, with the aid of zone formation under the influence of centrifugal force, that the viscosity of protoplasm suffers a sudden considerable increase with subsequent decrease after a few minutes, during the formation of the first and second polar body as well as in the beginning of mitosis. Increase as well as decrease of viscosity has an inhibitory effect on cell division. Anesthetics in weak concentrations and hypertonia reduce the viscosity and arrest the development of the fertilized eggs completely. Hypertonia and hydrocyanic acid increase the viscosity and also inhibit the development, in that the division makes no progress although the formation of a spindle shaped figure can be observed initially. *It is evident from the above that different physiological conditions of cells are closely related to different physicochemical conditions.*

ELECTRICAL PHENOMENA IN CELLS

Isolated cells are known to possess an appreciable charge. Blood cells and bacteria have a negative, paramecium a positive charge. This charge is easily influenced. The charge of the blood cells can be increased by anions, and reduced by cations, particularly by multivalent ones, such as Al, Fe, La. The cells are discharged or their charge is even reversed (Hoeber). Blood cells retain their charge in NaCl as well as in sugar solutions; it is, therefore, partly characteristic (proteins are negatively charged), partly acquired.

What is the rôle of the intracellular ions in the process of charging the blood cells or in the electric phenomena occurring in the cells generally? It has been demonstrated that the electric conductivity of serums is reduced by the addition of red blood cells in the same way as by the addition of

[34] Heilbronn, *Jahrb. wiss. Botanik,* **54** (1914).
[35] Heilbrunn, L. V., *J. Exp. Zool.,* **34** (1921).
* See paper by Heilbrunn in this volume. *J. A.*

sand. How does this compare with the fact that the cells contain ions which theoretically could participate in the transportation of the current? Since the cells are non-conductors, the ions are apparently prevented from leaving the cells and conducting the current outside of them. It can, however, be proved that blood cells have an inner conductivity (Hoeber). The capacity of an electric condenser can be increased by introduction of a conductor. A comparison between a blood cell suspension and NaCl solutions of various concentrations in the condenser capacity experiment, shows that the charge of blood cells is equal that of an 0.1-0.4 per cent NaCl solution. It follows that blood cells possess an *inner conductivity* or that potential differences, *i.e.*, e.m.f. can be produced in them by ions changing from the free to the cell-bound state and *vice versa*. Similar electric phenomena can be observed in the cells of living tissue. When a current is passed through a plant or animal organism, there is no agreement with Ohm's law; the resistance increases steadily while the e.m.f. remains constant and the rate of increase is the larger, the smaller the e.m.f. Moreover, the resistance is smaller for alternating than for direct current (Gildemeister, see below). The variability of the resistance, *i.e.*, its increase during the passage of the current, can be a result either of capacity or of polarization. Gildemeister has shown that the increase of resistance is caused by *polarization*. The cell of the skin—the subject of Gildemeister's investigation—is capable of producing electric energy. How can it be evoked? (1) By transformation of chemical energy. (2) Through differences in ion concentration (diffusion potentials). (3) Through phase boundary forces, *i.e.*, differences of potential, set up in the boundary surfaces of two immiscible phases. (4) By ion adsorption.

In order to understand the physiological significance of electric currents in the tissues, it is necessary to recapitulate certain fundamental electrophysiological facts, known since the time of Dubois-Reymond and Hermann. We know of the muscle that: (1) an injured part is always negatively charged against an uninjured one (current of rest), (2) an excited part is negatively charged against a non-excited part (action current), (3) a cooler part is negatively charged against a warmer one (thermo-current). An explanation for these differences of potential can be found in the fact that injured or irritated muscle parts are the site of acid formation. The acid migrates from the site of production. Since the velocity of the H-ion is higher than that of the anion, the injured part becomes negatively charged. Similar currents were observed in nerves, in skin epithelium and glands (see especially Gildemeister, Ebbecke).

The charge of the cell colloids and its variation play an important part in the *water economy* of the cells.

We know that the charging of proteins runs parallel with hydration: charged proteins are hydrated. *Electro-osmotic transportation of water* in the cells [30] plays perhaps an equally important part. Capillary walls are capable of adsorbing either cations or anions according to their original charge and thus becoming charged against water. If there is a difference of potential on the two sides of a diaphragm made up of capillaries, a transportation of water through the capillaries will take place (Perrin and others). A collodion membrane, for instance, is such a capillary system. The exchange of water and salt through such a membrane, *e.g.*, a collodion bag, naturally takes place only as long as there is a difference between the inside and

[30] See especially Bethe, *Arch. ges. Physiol.*, 63 (1916).

outside concentration. It has been however shown particularly by Jacques Loeb [37] that this rule applies exactly only to higher salt concentrations. In lower concentrations, at about 0.001 *N* the charge of the membrane has a considerable influence on the migration of water. If the collodion membrane is for instance filled with an 0.001 *N* solution of sodium citrate or of any other salt with multivalent ions, the outside water will pass into the membrane, since the inside surface of the membrane is charged negatively by the citrate. The water being positively charged against the capillary walls of the membrane migrates in the direction of the higher negative charge, in this case the inside. If, however, the membrane contains a calcium chloride solution of similar dilution, the inside of the membrane acquires a positive orientation and the water will diffuse to the outside, presenting the peculiar phenomenon of a water migration from a salt solution toward pure water accompanied by an increase of concentration. These phenomena are likely to occur also in cells.

All these phenomena justify the assumption that changes of concentration in the cells and consequently potential differences arise from the different binding capacity of different cell colloids for the different ions. These changes of concentration are on the other hand also dependent on the outside conditions. Slight alterations of the latter are capable of modifying the nature and distribution of the charge and thereby the functioning.

We have seen that the processes occurring in the protoplasm are influenced by the principal factors determining the state of colloids and alterations therein. These factors are: hydration, surface tension, difference of potential We can now proceed to the study of our main problem: to what extent can the effect of therapeutic agents on the cell functions find an explanation in colloid chemistry?

III.

The interrelation between colloid chemistry and pharmacology can be considered from the chemical or the pharmacological point of view. The first method would consist of an investigation of the influence of various substances on the individual and combined cell constituents and of correlating the results. At the present time success with this method appears to me to be remote. Preliminary steps in this direction have been already taken. They are represented by the numerous investigations of the effect of anesthetics on individual colloidal systems, their dehydrating effect on flocculated fibrin (Kochmann,[38]); their depressing effect on surface tension of widely differing colloids (Traube [39]); their precipitating action on colloids (Goldschmidt and Pribram [40]; Warburg and Wiesel [41]); the decrease of dispersity by the action of digitalis (Pietrkowski [42]); the promoting effect of caffein on albumin swelling (Handovsky [43]); the specific globulin precipitating action of arsenic compounds (Handovsky [44]). All these investigations have been used with more or less justification for the explanation of the pharmacological action of these substances.

Even the influence of poisons on processes in the inorganic domain were

[37] Loeb, Jacques, *J. Gen. Physiol.*, since 1918
[38] Kochmann, in Heffter, "Handbuch der experimentellen Pharmakologie," Bd. 1, **469** (1923).
[39] Traube, *Arch. ges. Physiol.*, since 1913.
[40] Goldschmidt and Pribram, *Z. exp Path. Ther.*, 6 (1909).
[41] Warburg and Wiesel, *Arch. ges. Physiol*, 144 (1912).
[42] Pietrkowski, *Biochem. Z.*, **98** (1919).
[43] Handovsky, *Biochem. Z.*, 25 (1910).
[44] Handovsky, *Biochem. Z.*, **25**, 526 (1910).

systematically studied, since the inhibition of catalytic reactions by organic poisons became known by the celebrated experiments of Faraday [45] in 1849 and Schoenbein in 1843. These investigations became of immediate avail for pharmacology after Bredig and Ikeda established the analogy between the poison of colloidal metals and of ferments from the kinetic point of view. The action of heavy metals and organic poisons on various enzymes was recently experimentally studied by v. Euler.[46] Rona and his students [47] attribute considerable biological significance to the fact that the effect of a given poison varies with the ferment. The behavior of invertase, for instance, toward quinine is different from that of the normal blood lipase, and even lipases of different origin are differently affected by quinine.

We shall therefore choose the second method for the detailed discussion and shall try to indicate how the various therapeutic effects can be summarized from the point of view of colloid chemistry.

We have seen that colloid chemistry rests on three fundamental bases: (1) The interconnection between individual colloids, that is, the "colloidal structure," (2) the importance of ions for these colloidal systems and the importance of the colloidal structure for the state of the ionogenic components of the cells, (3) the importance of surface active substances.

We know little about the interconnection of the individual colloids. A certain insight into the structure of a compound colloid is afforded by the knowledge of its decomposition products, *e.g.*, of the jecorin of the liver, which consists of protein, lipoids, and glucose, further by the study of the colloid mixtures represented by the ferments. Whatever the actual condition of the colloids in mixtures may be, we know that it is different from that of the artificially isolated constituents.* This is borne out particularly by the investigation of lecithin-protein mixtures by Handovsky and Wagner.[48] It was demonstrated in this work that lipoids extracted with ether from bovine and horse serum precipitate within 24 hours the serum from which they are derived, regardless of whether it is untreated or dialyzed, whether lipoid free or still containing lipoids. The precipitation increases proportionately with the lipoid concentration. The state of lipoids in aqueous emulsions apparently differs from that in the serum. Electrolytes prevent the flocculation, their inhibiting action increasing in the order: $NH_4 > Na > Ba (= Mg = SCN) > SO_4 > Cl$. Acids (which precipitate lipoids even in low concentrations) are capable of inhibiting the lipoid-protein precipitation, the active concentration depending on the proportion of the two constituents. At higher acid concentrations, however, the lipoids are precipitated and float on the surface of the solution. Bases have also the faculty of stabilizing lipoid-protein mixtures. The action of brain and egg lecithin on serum albumin is similar to that of serum lipoids. The investigations of Bechhold and students [49] on cholesterol-lecithin mixtures are to be mentioned in this connection. *The facts compel us to assume that the manner in which the colloids are united is an essential factor in cell function.** The detailed studies of Willstätter and Stoll [50] have

[45] Faraday, "Researches in Electricity."
[46] Euler, *Fermentforschung*, 3 (1920).
[47] Rona and students, *Biochem. Z.*, beginning with Vol. 111.
* See papers by F. Bottazzi, D. T. MacDougal, and Bridges and Alexander in this volume; also paper on colloidal protection by Alexander in Vol. I of this series. *J. A.*
[48] Handovsky and Wagner, *Biochem. Z.*, 31 (1912).
[49] Bechhold, *Münch. Med. Wochschr.* (1921).
* See also paper by A. Lottermoser, Vol. I this series, on irregular zones of precipitation, and the effect of order and rapidity of mixing reacting solutions. Colloidal protection is an ever-present factor in living things. *J. A.*
[50] Willstätter and Stoll, "Untersuchungen ueber die Assimilation der Kohlensaure," Berlin, 1918.

also demonstrated that a certain colloidal state of chlorophyll is the prerequisite of carbon dioxide assimilation.

Unpublished extensive series of investigations by Handovsky, Bosse and Lohmann on the colloido-chemical structure of blood serum throw some light on the variability of colloidal structure. The authors succeeded in demonstrating that the firmness with which cholesterol is bound depends on the proportion of protein substances, and that the ease of extraction of cholesterol is proportional to the albumin content, regardless of the quantity of cholesterol in the serums. The extraction of cholesterol is also facilitated by shaking the serum with kaolin and animal charcoal. The ratio of the proteins can also be modified. It was, *e.g.*, observed that the proportion of albumin to globulin is changed by allowing ether to trickle slowly through serum. The stability of sera with regard to modifying influences decreases as a rule, with the increasing albumin content.

We are compelled to assume that similar conditions prevail in the cell, that the alteration of one constituent, for instance, a change in the state of cholesterol or lecithin, or the swelling of one, the dehydration of another protein constituent results in an alteration of the total structure, and hence in a modification of the faculty for binding water and salt, and of the reactivity of the cells.

Surface active substances have sometimes a considerable influence on the state of colloids. Kruyt and van Duin [51] have observed that under certain conditions they accelerate the precipitation of colloids by electrolytes. In presence of camphor or thymol, ferric hydroxide is precipitated by one-third of the NaCl concentration required in the absence of these agents.

The rôle played by ions appears comprehensible when viewed in the light of numerous observations on inorganic colloids. We know that *electrolytes are extremely sensitive regulators of the colloidal state.* The action of the cations is opposed to that of the anions, because of their different charges. Whether one or the other ion dominates, depends on the sign and strength of the charge of the colloid, and on the effectiveness of the ion. In the case of the strongly charged ions of the heavy metals, the electrochemical influence dominates regardless of the nature of the colloidal system. The ions increase the charge of electropositive colloids, and discharge electronegative ones. Among the latter, the hydrophilic colloids require much higher ion concentrations than the hydrophobic. The bivalent cations of the alkaline earths have a less pronounced effect than the heavy metals, but a stronger one than the univalent alkali ions. They present interesting, individual differences for the different colloidal systems. The alkali salts act chiefly electrochemically (charging or discharging), on hydrophobic colloids; in hydrophilic colloids they mainly influence the hydration. With regard to their action on hydrophilic colloids, the alkali ions and the anions can be arranged in the well-known Hofmeister series. This series reappears with slight deviations in all physiological processes, *e.g.*, in the effect of salts on the excitability of muscles, the production of the current of rest, the reduction of ciliary movement.

The mutual relation between colloids and ions manifests itself either in the loss of the specific effects of the ion, or in certain modifications of the properties of the colloid (electric phenomena, surface tension). The influence is therefore mutual. Either both ions of the electrolyte are bound; or only one ion is bound and communicates its charge to the colloid; so that a molecularly dispersed ion is replaced by a colloidal one; or an interchange of ions takes place through the substitution of an ion fixed by the colloid by another ion; or, finally one ion is bound and discharged, making hydrolysis necessary for the neutralization of the other ion. The ions need not necessarily be bound; their action can be indirect, for instance by dehydration.

[51] Kruyt and van Duin, *Kolloidchem. Beihefte*, 5 (1914).

We have seen in the case of muscle and nerve irritability (*supra*) that it is by no means immaterial whether the ions in the cells are bound or free. The experiments on the lecithin-protein mixtures, reported above, show that the ions affect not only the state of the individual colloids, but also the colloidal structure.

Our next question is: Can the state of the colloids·within the cell be affected by ions?

The sensitiveness to poisons is a fairly good measure of this influence, and we shall first discuss the modifications produced by ions in the sensitiveness of cells and tissues to poisons. The dependence of the sensitiveness to poisons upon the ion medium is demonstrated particularly clearly by the influence of Ca and K ions on the poisonous effect. The Ca ion which increases the tone of the heart muscle, also increases the effect of strophantine, digitalis and adrenaline on the frog heart (Loewi,[52] Handovsky [53]). It abolishes the tone-depressing action of arsenious acid, quinine and chloral hydrate (Zondeck),[54] of cocaine (A. Mayer),[55] and of camphor (Handovsky).[56] The K ion which has a depressing effect on the heart muscle tone, enhances the effect of muscarine, philocarpine (Loewi),[57] arsenious acid, quinine, chloral hydrate (Zondeck),[54] cocaine (A. Mayer).[55] The K ion augments the electric conductivity of the peripheric frog nerves and the paralyzing peripheric action of urethane (Handovsky and Zacharias).[58] Ca enhances the tone-increasing effect of pilocarpine and atropine on the skeletal muscle of the toad (Loewi and Solti).[59]

In his analysis of the Ca-cocaine antagonism, A. Mayer [55] points out that cocaine inhibits blood clotting (Zak),[60] that its anesthetic action is inhibited by lecithin because it is bound by lecithin (Storm van Leeuwen),[61] and finally that a precipitation of a lecithin emulsion by Ca is inhibited by cocaine. Mayer concludes that calcium and cocaine displace each other in the combination with lecithin and that their functional antagonism is related to this fact. In this case the colloid upon which the antagonistic action takes place is known. The same can not be stated with an equal degree of probability for the other discussed cases. We can however assert, that *the sensitiveness of cells to poisons is influenced by the ion milieu* in a similar manner as is the state of inorganic colloids, and direct our attention toward the question whether there is also a relation between the state of protoplasm and its function.

We are fairly well informed on this relation as far as the physiological borderline cases are concerned; the anesthetized cells on one hand and the stimulated cells on the other. The exchange between the inner content of the cells and its environment is hampered in anesthetized, enhanced in stimulated cells. We shall call the ability of the cell to control the exchange of matter and energy its permeability. A complete theory of permeability was first advanced by Pfeffer,[62] a theory, which until the present day forms the basis of our investigations.

Pfeffer was the first to realize that dissolved substances can penetrate

the cell by two different means : (1) through the colloidal particles themselves by combining with them, (2) through the interstices occupied by fluid. It was pointed out already by Pfeffer that the width of these interstices is variable, which accounts for the inability of certain easily dialyzing substances to penetrate the cell. The fundamental part of this theory has not lost its validity for modern colloid chemistry. It has been enlarged by finding that permeability also depends on the physiological condition (Bernstein, R. S. Lillie, R. Hoeber).*

We shall present this evidence. Let us consider first the narcotized cell in which the permeability is lowered. The observed physiological, physical, chemical and colloido-chemical changes are presented in the following table :

TABLE II. *Narcotized Cell.*

Physiological Changes	Physical and Chemical Changes	Colloido-chemical Explanation
Lowered function.	(1) Decreased metabolism. (2) Lowered surface affinities; adsorption displacement even of water; dehydration, diminution of volume. (3) Increase of viscosity. Increase of polarity; the ions are bound more firmly by the cells.	Decrease of the degree of dispersion.

From the above, the assertion seems justified that in anesthesia the degree of dispersion of at least several cell colloids is modified. The analogy with simple colloidal processes permits us, furthermore, to attribute to this fact the observed physical and chemical changes. The underlying observations were made partly on isolated cells and partly on cells with simple functions such as epithelial cells.

The state of excitation of cells cannot be fixed with the same degree of accuracy, since excess excitation often impairs the function and the result is an overlapping of the two conditions.

However, the observations of Gildemeister, Ebbecke and Hoeber enable us to establish the following facts tabulated below:

TABLE III. *Excited Cell.*

Physiological Changes	Physical and Chemical Changes	Colloido-chemical Explanation
Intensified function.	(1) Increase of exchange. (2) Lowered viscosity. (3) Decreased polarity (liberation of ions from their combination with the cell colloids).	Increase of degree of dispersion.

The correlation between excitation and the increase of the degree of dispersion is evidenced chiefly by the results of studies of lowered polarity,

* See papers by Lillie and Hoeber in this volume.—*J. A.*

or increased conductivity of stimulated sweat glands in the so-called psycho-electric reflex. Gildemeister,[63] to whom we are especially indebted for these results, has demonstrated that the increase of conductivity appears simultaneously with the action current, *i.e.*, before the beginning of the specific activity of the cells. This proves that the "hole" in the cells in the sense of Bernstein's (1871) and Hoeber's membrane theory [64] is produced by excitation, that the increase of cell permeability is a result of excitation, not of cell activity.

Ebbecke and Hecht [65] have observed the same increase of conductivity in electrically stimulated plant cells. It was moreover established by Hoeber and Banus [66] that the cells thus treated are capable of taking up sulfonic acid dyes and salts during the passage of the current, which they cannot do under normal conditions.

The foregoing demonstrates in a convincing manner that the physical and chemical changes which take place in anesthetized cells on one hand and in excited cells on the other, are opposite and that they are attributable to contrary colloidal alterations, *i.e.*, diminished dispersity in the anesthetized, increased in the stimulated cell. During the period of life the colloidal state of the protoplasm oscillates between these two conditions.*

While the interpretation of the interrelation between colloidal condition and cell function is comparatively easy in cells with primitive function, it becomes more difficult in the case of muscle, especially as the different kinds of muscle (heart, skeletal, smooth muscles) react quite differently even in higher animals, and an entire set of processes is necessary to elicit a muscle contraction. We shall therefore refrain from a detailed discussion and only mention as an instance the veratrine action as it was established by Riesser and Neuschloss.[67] The interpretation of these authors has been partly opposed by Loewi and Solti.[68]

There are undoubtedly cells with primitive function, isolated as well as in the living tissue, in which a relation between permeability and function, in the above sense, exists. A decrease in permeability is the result of a decrease in dispersity of the colloidal protoplasm and *vice versa*.

Theoretically such a decrease in dispersity can be produced in several different ways,[69] namely by

    (1)   change of degree of hydration.
    (2)   direct change of the degree of dispersity.
        (a)   decrease of surface tension.
        (b)   precipitation.
        (c)   sol-gel transformation (gelation).
    (3)   changes in the structure of the compound colloid.

There are different kinds of impairment of cell functions, and we are with a certain degree of probability justified in assuming that each inhibition of function and diminution of permeability is attributable to one of the above

mentioned mechanisms concerned in the diminution of dispersity (Handovsky and Masaki [70]). These substances which are capable of depressing the cell functions can be divided into four groups: (1) anesthetics, (2) salts of heavy metals, (3) cane sugar, (4) tanning substances.

We have already discussed the anesthetics in detail and stated that they diminish the permeability and surface tension which results in adsorption displacement and dehydration. Results of other investigations make it probable that the action of narcotics is initiated not in all colloidal surfaces, but particularly in the lipoids (Meyer's and Overton's theory of anesthesia). The decrease of permeability and the impaired function caused by anesthetics would, according to this conception, consist primarily of a displacement of essential colloids from the cell lipoids, among others water. The latter is liable to affect the ion transportation, etc., but all these interpretations are still to a large extent hypothetical.

The effect of *salts of heavy metals* has been studied chiefly on plant cells. Szüsz [71] has demonstrated that the penetration of iron ions or of neutral red into the cells of algae, is prevented or delayed by a previous treatment of the cells with dilute aluminium salt solutions. He could furthermore prove that even very small amount of Al-ions cause gelation throughout the entire protoplasm, as manifested by the impossibility of separating the chloroplasts, for instance, by centrifuging. Gelation naturally decreases the dispersity of the protoplasm, and consequently its faculty to react with surface active substances.

The action of cane sugar was similarly interpreted by Handovsky [72] as a gelation of protoplasm. In red blood cells cane sugar causes dehydration, decrease of volume and of sensitiveness to hypotonic solutions. This effect increases with time as was shown by I. Bang.[73] This recalls the fact that the setting of a jelly is also gradual. Briefly, the effect of cane sugar on protoplasm can be interpreted as gelation, although here the evidence is not as strong as in the case of the salts of heavy metals. The assumption is supported by the analogy with the gelatinizing action of cane sugar on gelatin. Gelation would result in a lowered dispersity of the protoplasm, and consequently of its faculty of binding surface-active substances. This is in harmony with the findings of Handovsky to the effect that in cane sugar solutions, much higher concentrations of poisons are required to produce hemolysis of red blood cells, than in physiological salt solutions. The poisons are bound in smaller amounts. In addition Hoeber and Memmesheimer [74] have proved in a direct manner that the binding capacity is impaired.

The mechanism of the action of tanning substances was more recently studied by Handovsky and Masaki [75] and Handovsky and Heubner.[76] The investigation of the action of tannin on red blood cells revealed that this substance effects changes even in concentrations as low as 0.002 per cent, the nature of the changes being: (1) at very low concentrations inhibition of oxygen consumption and of the methemoglobin formation, provoked by nitrites. (2) At somewhat higher concentrations increase in volume, acceleration of red blood cell sedimentation, agglutination, appearance of in-

homogeneous specks, brilliant in the dark field. (3) At a higher concentration (about 0.02%) acceleration of methemoglobin formation, spontaneous hemolysis on shaking. The most comprehensible interpretation of these effects is a partial coagulation on the surface, due to the strongly dehydrating, colloid-precipitating action of tannin. The peculiar significance of ions for the extent of the tannin action, the inhibitory effect of OH-ions and particularly the favorable effect of Ca-ions on the derangement of homogeneity, and the inhibiting effect on agglutination, are strongly in favor of such a colloido-chemical process. The diminution of the degree of dispersion due to precipitation also accounts for the fact that in previously treated cells, hemolysis by saponin is retarded or requires a higher concentration than in untreated ones. The modification of the action of poisons by tannin could also be demonstrated in other kinds of cells. A cocaine concentration of 1 : 95 000 just produces an appreciable depression in the irritability of the sciatic nerve of the frog. After treatment with tannin, a concentration of 1 : 25 000 is necessary to produce the same effect. The stopping of the frog heart by strophanthine can be materially delayed by a preceding treatment with tannin. Thus we encounter everywhere the same action.
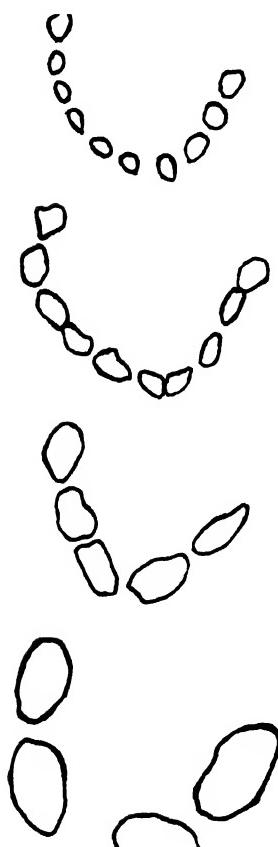
The behavior of tannin is of such instructive value for the relation between the colloido-chemical and pharmacological effects, that it deserves a schematic illustration (FIG. 1).

The importance of the colloidal state of the cell for the pharmacological effects can be summarized in the following conclusion: We have various means at hand by which to eliminate the matter and energy exchange between the cells and the surrounding medium, and thereby to lower the response of the cells to stimuli and to impair their function. These influences are brought about by various colloidal mechanisms.

FIG. 1.

As was pointed out at the beginning this effect on the cells gives no information with regard to the therapeutic applicability of the individual substances.

It can be presumed that the colloidal state of the protoplasm has an essential influence even on the more finely differenciated functional modifications. But our actual knowledge of these phenomena is scant.

Our experiences regarding the relation between colloidal state, function and pharmacological action can be illustrated by the diagram (Fig. 2).

IV

We have discussed, until now, the dependence of the effect of therapeutic agents on the functional and colloidal condition of cells and tissues. The

Function

Different for different organs

see: action of adrenaline
on sympathico-vagotonic hearts

ionic medium

Different for
different poisons
(see above)

sensitiveness to
poisons

see: e.g. anesthesia and stimulation

Different for different colloids

see: action of tannin
cane sugar, heavy metals
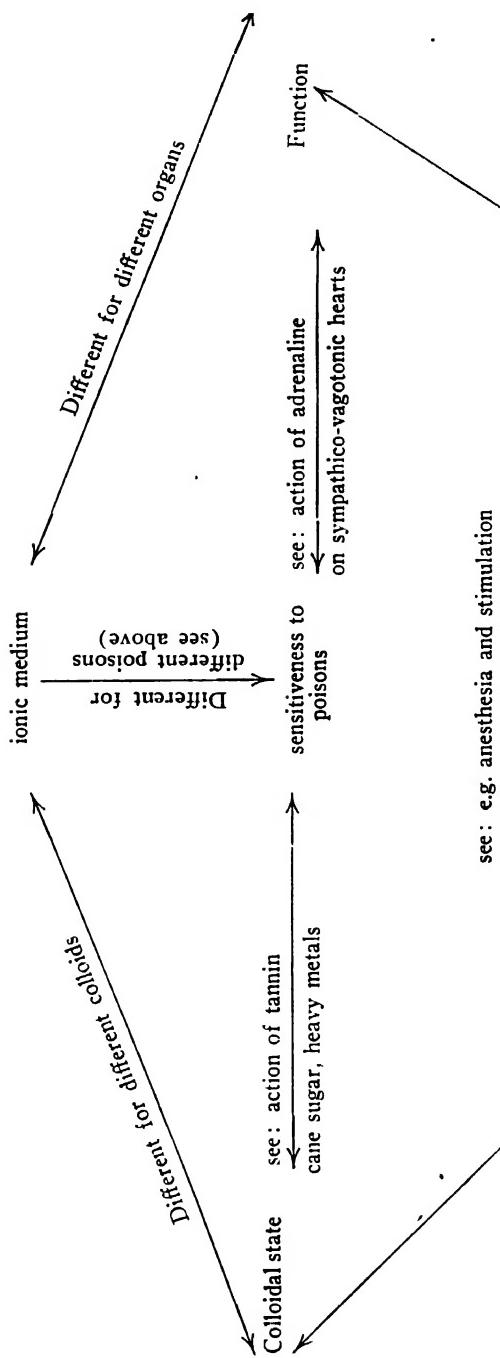
Colloidal state

FIG. 2.—The relation between colloidal state, function and pharmacological action.

importance of the *condition of the therapeutic agents themselves* must not be disregarded. All agents which act after being absorbed, pass through the blood stream, and it is therefore necessary to point out that the blood itself exerts a pharmacological action. It was first discovered by O'Connor [77] that blood serum has a vasoconstrictor effect.* It also stimulates the intestine, uterus and heart. Handovsky and E. P. Pick [78] have made a detailed investigation of the action of serum and have made the following observations: (1) The vasoconstrictor effect increases materially with time even if autolytic processes are avoided. (2) The dialysate is ineffective. (3) The effect is produced by the albumin fraction. (4) Shaking of serum with indifferent adsorbents (kaolin, diatomaceous earth, fibrin) increases its vasoconstrictor action.

The authors assume that these effects of serum are the result of a *progressive separation of the colloids of blood-fluid*.

At the present time we believe we have gained a somewhat better understanding of these effects.

We shall consider first the effects of serum on the animal as a whole.

(1) We know that injections of proteins and protein cleavage products have a powerful effect on the entire organism (humoral change, activation of protoplasm in Weichardt's sense). Among these phenomena are: polynuclear leucocytosis, rise of temperature, localized focal reactions.

(2) It was proved by Doerr and Berger [79] that protein injections cause an alteration in the colloidal structure of the blood-fluid. They produced in the rabbit by a single protein injection the following reactions recurring with remarkable regularity: first a decrease, then an increase, then again a decrease and after 60-80 days following the injection again an increase of the total protein content which was finally restored to normal. In the globulin fraction: latent period, decrease of very short duration, rapid increase, subnormal values. Albumin content: latent period, increase (maximum in 60-100 days following the injection) return to normal. Summary: *hyperglobulinemia,* followed by *hyperalbuminemia.*

(3) It was repeatedly observed, especially by Freund and Gottlieb [80] that injections of protein substances, *e.g.,* caseosan, are followed by a change in the sensitiveness of certain organs to poisons. There is evidently a relation between the sensitiveness and the changes in the blood.

(4) Handovsky, Bosse and Lohmann have found in an extensive series of experiments, soon to be published, that the firmness with which cholesterol is bound in serums, has some relation to the ratio of the individual protein substances, serums rich in albumin retaining cholesterol less firmly than those with a high globulin content.

The addition of very small quantities of sugar or salts, *e.g.,* 0.2 g. to 100 cc. serum affects the firmness of cholesterol fixation, the effect depending on the composition of the serum and the quantity of the substance added.

(5) Under certain circumstances injections of such minute quantities of sugar or table salt into humans are followed by considerable changes in behavior, drop in blood pressure, increase in polynuclear leucocytes (Erich

[77] O'Connor, *Arch. exp. Path. Pharm.,* 67 (1912).
* See also the work of A. Krogh and collaborators, e.g. in "The Anatomy and Physiology of Capillaries," Yale Univ. Press, 1922. The posterior pituitary hormone regulates the tonus of capillaries, a highly purified hormone produced by J. Abel (Johns Hopkins) being effective in one part in 18,750,000,000. *J. A.*
[78] Pick, E. P., *Arch. exp. Path. Pharm.,* 71 (1912).
[79] Doerr and Berger, *Biochem. Z.,* 131 (1922).
[80] Freund and Gottlieb, *Arch. exp. Path. Pharm.,* 93 (1922).

Meyer).[81] After the injection the serum of these individuals exhibits changes in the colloidal structure, similar to those obtained in vitro, *i.e.*, weaker fixation of cholesterol (Handovsky and Lohmann).

Consequently the phenomena elicited by the intravenous injection of minute quantities of salt or sugar are closely related to the change in the colloidal structure.

(6) Certain blood constituents, such as cholesterol or lecithin are by themselves alone capable of influencing the sensitivity of organs to poisons. This was demonstrated especially by Storm van Leeuwen [82] for the action of pilocarpine on the intestine of the cat. This effect is intensified by cholesterol and diminished by lecithin.

With these relations as a basis we believe we are justified in advancing the following theory of serum action:

The cells are surrounded by an envelope of constituents of the blood-fluid. The composition of this envelope is different and varying; sometimes the pseudoglobulins prevail, sometimes the euglobulins, or cholesterol, lecithin, etc. This depends on the actual momentary composition of the blood-fluid (par. 4), *which is also in vivo easily modified* by small alterations in the ion content (par. 5). These alterations of the colloidal structure, which during life very likely occur continuously although irregularly, result, in their turn, in a modification of the sensitivity to poisons (and hormones), of the exchange of matter, of the colloidal structure of the cell colloids and hence of function. *The blood plays, accordingly, a definite and perhaps not inconsiderable part in the extent of the action of poisons.*

V

There is a great analogy in more than one regard between the pharmacological action of protein substances and that of other colloidal solutions.

The phenomena evoked by injections of silica, starch, or gum solutions, are, according to Luithlen,[83] the same as those following protein injections, apart from the specific action of the latter (polynuclear leucocytosis, fever, inhibition of inflammation). It is probable, although it has not yet been investigated, that they act through the derangement of the colloidal blood structure.

Colloidal metals act similarly. They combine the effects of colloids and metals, as was shown by O. Gros.[84] Gros has observed ascending paralysis in frogs following the injection of collargol. This is a typical silver effect. In the rabbit the effect of silver chloride suspensions varies according to the size of the particles; it is, however, always a characteristic silver effect (see also A. J. Clark).[85] The intensity of the effect depends on the rate of liberation of silver ions. Here the blood acts as a protective colloid. The action of silver chloride is to some extent also of a general colloidal nature as evidenced by leucocytosis, rise of temperature and the adsorbing, hence detoxifying effect, on other simultaneously injected poisons. That the lethal dose of metals is much higher in the colloidal than in the crystalloidal state is self-evident. Recently Heubner [86] succeeded in producing in cats, by means

[81] Meyer, Erich, "Verh. Kongr. D. Ges. innere Med., 1924.
[82] van Leeuwen, Storm, *J. Pharmacol.*, 17 (1920); 18 (1921); *Arch. exp. Path. Pharm.*, 90 (1920).
[83] Luithlen, "Vorlesungen ueber die Pharmakologie der Haut," Berlin, 1921, p. 31.
[84] Gros, O., *Arch. exp. Path. Pharm.*, 64 (1911); 70 (1912).
[85] Clark, *Brit. Med. J.* (1923).
[86] Heubner, *Klin. Wochschr.*, 2 (1923).

of colloidal calcium phosphate, barium sulfate and silica protected by gelatin, the ascending paralysis observed by Gros for silver chloride suspensions and the inhibitory effect on inflammation which was found by Luithlen for a number of colloids. We have no explanation for these phenomena at the present time.*

The colloidal conception has enabled us to penetrate relations of a general nature, principally *the relation between ion action, drug therapy proper and colloid therapy*. We have discussed in detail the relation between ion action and drug therapy. The relation between the former and colloid therapy is evidenced by the fact that, for instance, simple ions modify the colloidal structure of the blood, blood colloids are thereby transformed into foreign colloids, and the organism itself is thus to a certain extent compelled to produce foreign colloids, which may be directly introduced into the system in accordance with the principles of protein therapy.

Colloidality of Arsphenamine and Its Derivatives

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Arsphenamine (also known as Salvarsan and "606") is by far the most prominent of synthetic drugs.

Although not all syphilologists agree, the majority of physicians believe that the administration of arsenic in the form of arsphenamine is more effective in the treatment of syphilis than are other organic arsenicals of earlier origin (such as sodium cacodylate).

Arsphenamine, and its derivative neo-arsphenamine, is supposed to have a higher spirochaeticidal power than other compounds of arsenic [1] and it is possible to safely administer considerably more arsenic as arsphenamine than in the case in almost all other compounds of arsenic, either organic or inorganic.

These therapeutic advantages and some of the toxic properties of arsphenamine are phenomena of the colloidal properties of arsphenamine.

That arsphenamine is a colloid is made clear by the following facts:

1.—Visibility of the particles under the dark field microscope. It is possible to observe the Brownian movement of the arsphenamine when dissolved both in acid and alkaline solution. The addition of a little H_2O_2 to the arsphenamine under examination causes the colloidal arsphenamine particles quickly to disappear. This is due to the fact that H_2O_2 rapidly converts arsphenamine into a crystallizable oxidation product ("arsenoxid") that exists in molecular proportions in the solution and is, of course, too fine to be visible in the dark field microscope.

Arsphenamine when kept for some days at room temperature in a vacuum, changes in color from a pale greenish yellow through orange to a deep cherry red. This change in color is due solely to a progressive colloidal agglomeration of the particles of arsphenamine. The red arsphenamine consists of larger colloidal particles that have a slower Brownian movement and are visible as such in the dark field microscope.

2.—Arsphenamine resists all attempts made to crystallize it. This is in keeping with its colloidal nature. When water is added to arsphenamine the powder imbibes the water, swells, becomes gummy and sticky and is slowly dispersed.

3.—Arsphenamine dialyzes very slightly. Only 10 per cent to 20 per cent of the arsenic in arsphenamine can be found to have dialyzed after dialysis for a week under an atmosphere of nitrogen and this dialyzable quota is mostly the crystalline oxidation product of arsphenamine that forms during the manipulation of the drug in the air while performing the experiment.

4.—Arsphenamine shows the Tyndall effect clearly. It is more plainly visible in the acid solution of arsphenamine than in the alkaline solution. Corresponding to this phenomenon, the particles of arsphenamine in acid solution

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[1] Ehrlich-Hata, "Die experimentelle Chemotherapie der Spirillosen," Berlin, 1910.

appear larger in the dark field microscope than do the particles in alkaline solution.

5.—Arsphenamine can be readily salted out by shaking with sodium chloride or some other neutral salts close to their saturation point. The acid solution of arsphenamine is somewhat more susceptible toward salting out than is the alkaline solution, a fact that further confirms observations given above showing that the acid solution of arsphenamine is less well dispersed than is the alkaline solution. The presence of neutral salts accelerates the aging of arsphenamine solutions. That is to say, arsphenamine solutions under a vacuum change in color (agglomerate) from greenish yellow to red much more rapidly if neutral soluble salts are present in considerable concentration than in case the concentration of such salts is low.

6.—Arsphenamine behaves very much like some proteins (e.g., casein) toward acids and alkalis. Arsphenamine is practically insoluble in water at the neutral point, whereas it "dissolves" in both acids and alkalis when they are present in sufficient concentration.

From the above facts it is apparent that a new conception of arsphenamine and its reactions must be formed.

Also the original idea that arsphenamine forms mono- and di-sodium salts and mono- and di-hydrochlorides must be substituted by the following view:

Arsphenamine is a colloid that is peptized by many strong acids or by caustic alkalis in solution.

The fact that arsphenamine can be completely "dissolved" by less than the stoichiometrical amount of either acid or alkali necessary to form the di-sodium salt or di-hydrochloride is a fact that can only be explained rationally on a colloidal basis. It might be suggested that the explanation of this phenomenon could be that if sufficient alkali or acid is added in stoichiometric amounts to form all of the mono salt and also some of the di-salt, the latter salt in turn dissolves the mono salt. This idea is, however, far-fetched and the whole phenomenon of "dissolving" arsphenamine is clearly an example of peptization by acids and alkalis.

It has been mentioned above that arsphenamine solutions kept in evacuated ampoules gradually undergo a progressive color change from greenish yellow to cherry red. Such age change was explained above to be due to a progressive agglomeration of the arsphenamine particles. This agglomeration is hastened by the presence of neutral salts and by heat. Inasmuch as the surface tension of a colloidal solution is believed to be a factor in regulating the tendency of the dispersed phase toward agglomeration, it seems reasonable to expect that substances that lower the surface tension of the arsphenamine solution ought to retard this color change.

It is a fact that when such substances of low surface tension (e.g., ethyl or methyl alcohol) are added to an evacuated aqueous solution of arsphenamine the rate of the color change is materially decreased.

Arsphenamine occasionally produces different kinds of dangerous manifestations when intravenously injected.

The two principal types of toxic symptoms are the so-called "nitroid reaction" and ordinary arsenical poisoning.

The "nitroid reaction" [2] is a typical serum reaction somewhat similar to anaphylactic shock, and is recognized as being a colloidal manifestation in the blood stream. It has been observed that insufficiently alkalinized arsphen-

[2] Berman, L., "Nitroid Crisis after Arsphenamine Injections," *Arch. Internat. Med.*, **5**, 217 (1918).

amine is very likely to produce it. It is also a fact that when arsphenamine is peptized by caustic soda it is advisable that this solution should set quietly for four or five minutes after the arsphenamine appears to have completely dissolved before making the injection, as otherwise a "nitroid reaction" may appear.

If examination be made by means of the dark field microscope of arsphenamine during the process of peptization by alkali, it will be found that even after the solution appears to have become clear to the naked eye the colloidal particles of arsphenamine are much coarser than they become subsequently after the alkali has acted further for several minutes. It is easily understandable that the tendency to produce a nitroid reaction should be greater by incompletely dispersed arsphenamine particles than if the arsphenamine is completely peptized by the alkali.

A fact about arsphenamine that has been puzzling lies in the amount of arsenic that can be safely injected as arsphenamine. This amount is considerably higher than can be given in most other compounds of arsenic (e.g., sodium cacodylate).

It is probable that the explanation exists in the fact that the colloidal arsphenamine is not poisonous and that oxidation in the body to a crystalline form of arsenic (arsenoxid) is necessary before the arsenic can manifest its well-known physiological effects. This oxidation process in the body requires several hours' time [3] and it is plausible that such crystalline (and active) arsenic is excreted as it forms at a rate sufficiently rapid to prevent toxic symptoms from appearing in the organism.

The ready oxidizability of arsphenamine is its outstanding characteristic and this idea regarding its toxicity is strengthened by the fact that if arsphenamine has accidently been oxidized it produces upon injection fatal arsenical poisoning although the actual amount of arsenic in the crystallizable arsenical compound so administered is the same as though it had been given as unoxidized colloidal arsphenamine. In short, the colloidal form of arsphenamine allows it to become slowly available to the body by gradual oxidation in the blood stream to a crystallizable compound of arsenic, thereby permitting the administration of a single large dose of arsenic that would otherwise be fatal.

A curious point mentioned by Erhlich [4] and others, is of interest in this connection. They found that the strength of a solution of arsphenamine required to kill the spirochaete of syphilis in a test tube or beaker (in vitro) has to be much higher than the concentration of arsphenamine necessary to kill the spirochaetes in the human blood stream (in vivo). It is probable that the blood plasma has the effect of forcing the colloidal arsphenamine particles into the interface between the spirochaetes and the blood plasma, thereby sufficiently concentrating the arsphenamine on the surface of the spirochaetes to produce fatal consequences of those microörganisms.

Silver arsphenamine also has colloidal peculiarities. This substance has been given a structural formula showing the silver existing in a stoichiometric relationship to the arsphenamine.

It is practically certain that silver arsphenamine consists of an insoluble

[3] "Elimination of Arsphenamine and Neo-arsphenamine in the Urine," F. Underhill and S. Davis, *Arch. Dermatol. & Syph.*, 2, 40 (1922).
[4] Ehrlich-Hata, "Die experimentelle Chemotherapie der Spirillosen," Berlin, 1910. p. 22.
Krantz, W., "Spirochatenkulturen in salvarsanhaltigem Nährboden," *Münchener med. Wochenschr.*, Dec. 29, 1922, p. 1782.

salt of silver (or possibly in some cases an oxide of silver), that has been peptized by arsphenamine and that no strict stoichiometric relationship exists between the silver and the arsphenamine in the sense of an organic combination. The following facts strengthen this view:

1.—Silver arsphenamine is visible in the dark field microscope and is hence a colloid.

2.—If silver arsphenamine be made by adding silver nitrate to commercial acid arsphenamine, the HCl present in the arsphenamine certainly forms AgCl, as it is chemically absurd to think that a large organic molecule such as arsphenamine would have a greater affinity for Ag than the Cl of the HCl present. Since AgCl is highly insoluble in water and yet silver arsphenamine is transparent (although brown in color), these apparently contradictory facts can become comprehensible only by the explanation that the AgCl is peptized by the arsphenamine as fast as the former precipitates in the arsphenamine solution.

When freshly precipitated silver chloride is added to an acid arsphenamine solution, a large quantity (more than half) of the silver chloride is peptized by the arsphenamine, the quantity so peptized depending upon how freshly precipitated the AgCl happens to be.

The peptizability of AgCl by acid arsphenamine is unquestionable, as is also the fact that AgCl is formed when $AgNO_3$ is added to ordinary acid arsphenamine.

The fact that this peptization is better if the AgCl is formed in the arsphenamine solution rather than added in a previously precipitated condition is in accord with other cases of peptization (e.g., glycerol and Fe $(OH)_3$, wherein the glycerol peptizes the Fe $(OH_3$ infinitely better if the Fe $(OII)_3$ is formed in the glycerol containing solution rather than added to it after having been previously formed.

Among the many papers dealing with the colloidality of arsphenamine, may be mentioned: J. Danysz, 1917, "Les Proprietesphysicochemique des produits du groupe des arsenobenzenes, etc." *Ann. Inst. Pasteur*, 21, 114-137; H. Bauer, 1919, "Kolloidchemische Studien in der Salvarsanreihe, etc.," *Arbeiten Inst. Exp. Therapie und Georg Speyer-Hause, zu Frankfort a/M.*, No. 8, p. 45 (Gustav Fischer, Jena); Z. Klemensiewicz, 1920, "Sur des proprietescolloidales des solutions aqueuses du salvarsan," *Bull. Soc. chimique de France*, 27, 820; C. N. Meyers, *J. Lab. Clin. Med.*, 7, 1912 (1921); Voegtlin, Johnson, and Dyer, Reprint No. 898 from *U. S. Public Health Reports*, Feb. 1st, 1924, pp. 179-195. Elias Elvove and Wm. Mansfield Clark have also studied the acid-base equilibrium of arsphenamine, and especially the pH of the solution injected. (*U. S. Hygienic Lab. Bull.* 135, March, 1924, which also contains J. M. Johnson's work on osmotic pressure). See also Hunter and Patrick on the physical and colloidal chemistry of arsphenamine, *J. Lab. Clin. Med.*, 10, (1925).

The Pharmacodynamics of Colloids

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I. Colloid Therapy

The physiological action of colloids has been studied in the majority of cases under the most erratic experimental conditions. The colloids used for the experiments were in some instances prepared by chemical methods, in others by mechanical or electrical dispersion. While some authors employed stabilized, sterilized, isotonic colloids, others disregarded these factors partly or entirely.

Only the investigations of Robin—unfortunately very few in number—carried out with non-stabilized, non-isotonic colloids, can be considered as a starting point for an experimental foundation of colloid therapy. As a result of his investigation, Robin arrived at the conclusion that all colloids studied have the same therapeutic properties regardless of the differences in their chemical nature; that they possess a curative effect in certain diseases (pneumonia, diphtheria) and are ineffective in others (tetanus, cancer, tuberculosis). Robin's view was at first opposed by other workers in this field. But in the course of time a more exact knowledge of the phenomena of the colloidal state permeated the medical world and lately Robin's simple theory began to receive attention on the part of other students, among which were Roger, Richaud, Nolf and others.

These merely empirical views did not, of course, satisfy the majority, still spellbound by the dogma of the strictly chemical specificity of colloidal actions.

A knowledge of the physiological effect of colloids is indispensable for an explanation of the mechanism of their action on the body fluids.

The therapeutic application of colloids originates in their antiseptic effect. It was pointed out as early as 1899 by Schlossmann and Baldoni. The recent researches of Brunner, Cohn, Bayer, and V. Henri, have brought very contradictory results with regard to this subject. C. Foà and Aggazzotti have undertaken a revision of the problem of the disinfecting properties of colloids, and have arrived at the following conclusions: Among colloidal metals, platinum, gold, bismuth, mercury and ferric hydroxide have no influence whatever on the growth of micro-organisms. Only finely dispersed silver and arsenic sulfide have an inhibiting effect. It is, however, to be borne in mind that arsenic sulfide always contains arsenic trioxide and that the latter is probably responsible for the bactericidal action. Similarly colloidal silver contains ionized silver and silver oxide.

Two facts seem to support the view that the antiseptic effect is attributable to the colloidal state: (1) Only finely dispersed colloids have an antiseptic effect; this would indicate that the physical condition of the colloid is of importance. It was, however, proved by Rebière that electrically prepared

colloidal silver consists of a suspension of silver micells in a medium containing dissolved silver and silver oxide. It was moreover shown by conductivity measurements and qualitative analysis that the amount of dissolved silver and silver oxide in coarse-grained solutions on one hand and in fine-grained on the other, is by no means equal, and that it is considerably higher in the latter. (2) It was observed by V. Henri that when the inter-micellar fluid of a fine-grained colloidal solution was separated from the micells by filtration, and added to a culture, it produced no antiseptic effect. This led Henri to the conclusion that the antiseptic effect is an inherent quality of the colloidal state. In fact, however, this experiment of Henri is not convincing. It affords no evidence that in the presence of broth culture the colloidal state of the silver is destroyed and that its flocculation is not followed by a partial or total dispersion of the silver complex. This experiment cannot be considered as conclusive unless carried out under different conditions, namely, by adding the colloidal silver to the sterile culture medium, subjecting the whole to ultrafiltration, and inoculating the filtered medium. On the other hand, it is well known that bacterial activity rapidly produces acids and bases, which very soon tend to destroy the colloidal equilibrium, causing the silver to pass into the ionized state. Thus infinitely small traces are capable of producing a bactericidal effect, a fact well known since the memorable researches of Rollin. The ultimate conclusion is that the bactericidal action of colloidal metals—using the term bactericidal in its current meaning—is more than questionable. A systematic study of the publications on this subject confirms the opinion that the growth-inhibiting effect of colloidal silver does not furnish a satisfactory explanation for all its physiological effects.

The catalytic action of colloids, for which the interest of the scientific world was awakened by the publications of Bredig,* was in its turn considered as a possible explanation.

To-day the catalyst theory of the colloidal action has lost much of its force. We know in the first place that there is no relation between therapeutic action and catalytic properties, and that the latter are relative and possessed almost exclusively by platinum and silver. This is borne out by the following table:

Amounts Exhibiting Like Catalytic Effect.

Pt	0.000 077
Os	0.000 023
Ag_2	0.000 1
$Fe(OH)_2$	0.002
Au	0.014
As_2O_3	0.02

There followed a period of researches of the *antilytic, antiflocculent or antitoxic* properties of colloids. In contrast to the formerly established opinion, C. Foà and Aggazzotti reached the conclusion that colloids have no effect on the toxins of tetanus, diphtheria and dysentery.

The *toxic* effects of colloids are still to be considered. Among the numerous researches on this subject, those of Foà and Aggazzotti deserve special mention, since these authors have attacked the problem from a general point of view. They have found that the injections of colloidal metals into the saphenous vein of the dog results in death, provided the dose is sufficiently

* See paper by G. Bredig in this volume. *J. A.*

large.   Death is attended by symptoms of sudden asphyxia due to acute edema of the lungs.   Necropsy shows a heart engorged with liquid, non-coagulated blood.   Hemorrhages in the organs are also invariably present.   The same symptoms and anatomo-pathological lesions appear under similar experimental conditions with finely dispersed silver, arsenic sulfide and other colloids. They are also present in clinical cases.   About one-half hour after the injection the reaction begins with a sensation of cold, soon followed by severe chills which may last about forty minutes.   The patient trembles, his teeth chatter like in the most severe attacks of malaria.   He suffers great distress and the perspiration is so abundant that the linen has to be changed repetedly.   Sometimes slight cyanosis of the face is present.   The temperature rises as high as 42.2° C., the pulse is accelerated.   Profuse perspiration begins about one hour after the onset of the attack and sometimes lasts several hours. Nervous patients occasionally present the picture of a slight and transient subdelirious excitation.   The normal condition is restored within a few minutes.

The determination of the lethal dose reveals the most striking lack of parallelism between the colloidal metals and the same metals in salt form.

What then is the mechanism of colloidal action?   Undoubtedly the chemical nature of the dispersed metal is of no importance at all.   A glance at the toxic effects of the salts of the metals studied clearly reveals this lack of relation. According to Ronyer the toxic dose of arsenic trioxide when given intravenously is 0.003 gram per kg. dog.   We know that 0.07 g. of this substance is required to cause death when introduced into the gastrointestinal tract. On the other hand, considerable quantities of arsenic sulfide can be introduced into the stomach without any ill effect, owing to its insolubility.   Richet cites experiments of Hillefeld who gave a rabbit 10 grams of this substance without producing serious sequelae.   Moreover a comparison of the symptoms of arsenic oxide poisoning with those associated with death following injection of colloidal arsenic sulfide, proves beyond doubt that the two phenomena are entirely different.   More instances can be adduced in evidence of this conclusion.   Arsenic in form of arsenobenzenes can be given in much higher doses than in form of inorganic salts.   The colloidal nature of the arseno-benzenes accounts for the lower toxicity.

In view of these facts we are necessarily led to the following conclusion: Colloids as such produce a specific action.   Death caused by the intravenous injection of any colloid is invariably attended by the same symptoms regardless of the chemical nature of the colloid.

Attempts were also made to find an explanation for the symptomatology and the death following intravenous injection of colloids.   The sudden drop of arterial pressure, the lowered temperature, the decrease of the respiratory capacity of the tissues and the promoting effect on oxidation processes were pointed out in this connection.   But all these phenomena *are the results and not the cause of shock*.   Shock ensues rapidly, sometimes suddenly.   The rapid onset is often followed by the complete disappearance of the most violent symptoms.   Necropsy invariably reveals the symptoms described by Foà and Aggazzotti: pulmonary emphysema with hemorrhagic areas; the heart is engorged with blood; clotting is considerably delayed, sometimes by fourteen hours; the blood picture is changed; there is leucopenia of the Le Fèvre de Arric type.   Symptomatology and lesions are characteristic of anaphylactic shock and of all shocks following introduction of a foreign sub-

stance into the circulatory system. Fatal shocks similarly result from the introduction of fine suspensions into the circulatory system. Thiele and Embleton have reported similar results produced by carmine, kaolin, etc. The data of these authors are unfortunately not exact with regard to the size of the introduced particles. It, however, follows from the researches of various authors, that the degree of dispersion of the metals is a dominant factor in their toxicity. Thus Foà and Aggazzotti have found that 0.002 g. of fine-grained colloidal silver is required to cause death, while the lethal dose of coarse-grained colloidal silver is much less. The same was found for electrically dispersed colloidal arsenic trioxide: 9 mg. As_2O_3 per kg. animal causes death, while coarse-grained As_2O_3 is fatal in doses as low as 1.5 mg. The very violent reaction following the injection of "collobiases" which are rather coarse suspensions can be interpreted in the same way. The coarser the suspension the smaller the quantity sufficient to produce embolism. There is a substantial difference between coarse suspensions, and colloids consisting of small or amicronic micells. The former are capable of producing intravascular clotting and multiple thrombosis, the latter never produce these emboli directly.

What then is the action of these colloids on the organism after they are introduced into the circulatory stream? The striking resemblance of the symptoms, induced Nolf to compare colloid shock with peptone shock observed by him after the introduction of large quantities of peptone into the blood. He has even maintained that the presence of protective colloids accounts for the effect of inorganic colloids. Although it appears quite possible that large quantities of peptone, when intravenously injected, may produce phenomena of shock by lowering the surface tension, yet the quantities of stabilizing colloids usually present in colloidal metals are too small to account by themselves in all cases for the violent reaction. Moreover, certain substances employed for that purpose, *e.g.*, gum or starch are harmless on first injection and have no marked influence on the surface tension. Their effect consists of an increase of viscosity finally tending toward the stabilization of the humoral medium. It is therefore logical to admit that there exists a colloidal action proper.

What is the nature of this action? In other words, what is the effect of colloids introduced into the blood on the colloids and the formed elements of the blood?

A review of the facts relative to the action of the colloids on each other and of electrolytes on colloids, will help us to form a definite opinion. This action is illustrated by the importance of the electrical charge. In this connection it is to be borne in mind that blood is an extremely unstable colloidal system, liable to be upset by the slightest variations, such as changes of temperature or the presence of substances normally absent from blood. Pathology furnishes examples of disturbances of colloidal equilibrium. The destruction of the colloidal equilibrium as the cause of paroxysmal hemoglobinuria *a frigore,* established by Widal, is the first instance of this kind. We have pointed out in 1921 that sunstroke presents certain analogies to paroxysmal hemoglobinuria *a frigore,* if we consider the importance of temperature changes in both conditions. In fact, it is to be borne in mind that the blood equilibrium is established at 37° C., not at any lower or higher temperature. On the other hand, dry diabetic gangrene, hemolytic icterus, uremia can be, at least partly, explained by the presence of substances which can be chemically identi-

fied and which upset the equilibrium of the blood colloids or of the formed elements. They cause death of the tissues either by establishing a defective permeability of cell membranes (dry gangrene), or by dissolving them (bile pigments in hemolytic icterus). The same disruption of colloidal equilibrium is associated with injections of lethal doses of colloids. This can be illustrated by the following experiment: 5 cc. of ferric hydroxide (conductivity 22.4.10⁻⁶ after 18 days' dialysis) is injected into the jugular vein of a guinea-pig. Death ensues with the symptoms described above. Examination of blood serum reveals a drop of 3-5 dynes in surface tension. The electronegative "globulins" have to a large extent acquired the opposite sign. It is obvious that the equilibrium is upset.

What is the nature of this disturbance? We shall see at the end of this paper that it is exactly the same as that observed in the phenomena of shock.

According to Iscovesco the "globulins," *i.e.,* the most unstable part of the serum, are electronegative. Our measurements, however, carried out under conditions under which electrolysis could not interfere with cataphoresis, have revealed that serum contains besides the negative globulins also electropositive globulins, although in a very small amount. This fact explains satisfactorily a number of phenomena: the disruption of the blood equilibrium by minute quantities of a positive colloid (ferric hydroxide), while relatively large quantities of a negative colloid are necessary to produce the same effect (Foà and Aggazzotti); the syndrome of paroxysmal hemoglobinuria *à frigore* as a result of a drop of temperature; the shock observed by Widal as a result of the introduction of large amounts of colloids.

Our investigation would not be complete without the study of the effect of colloids on the formed elements of the blood. This effect can be either direct or indirect: it is capable of furnishing an explanation for the main phenomena of life: agglutination, lysis, sensitization. The direct action of the colloid, which (this is an essential point) consists of micells and intermicellar fluid, is dependent on the physicochemical properties of both. The electric charge of the micells, the hydrogen ion concentration, the viscosity and the surface tension of the intermicellar fluid are of foremost importance, and have to be taken into consideration whenever the problem of the effect of colloids on the formed elements of blood is approached. It is intelligible, in fact, that a substantial decrease of surface tension, for instance that caused by soaps, bile salts, alcohols, ethers, narcotics and antiseptics, which gives rise to lysis *in vitro,* is capable of producing the same effect *in vivo.* The contrary effect, agglutination, would occur in case of an increase of surface tension. On the other hand, the electric charge of the ions of the intermicellar fluid is equally able to produce, in the human medium, an agglutination of the formed elements which apparently are always negatively charged.

This study based on an experimental physical foundation enables us to interpret colloid therapy as a therapy by shock. If this holds true for the natural and synthetic inorganic colloids, we ought to be able to prove our hypothesis for the organic colloids.

II. Protein Therapy

What is the mechanism of the therapeutic effect of protein substances?

Weichardt has attempted to determine this mechanism. The result of his researches, commenced in 1912, was the theory of "plasma activation," which

met with favorable comment in Germany. All the conceptions of this kind, including v. Gröer's ergotropic, "*i.e.* proteinotropic," therapy as opposed to "parasitotropic" or chemical therapy; Much's "stimulation of unknown immune forces"; Linding's "excitation of the tension of the organism," or finally the "pyrotherapy" of Paltauf, Bier or Konteschweiler—all these hypotheses although they differ in terminology have in common the lack of an exact experimental foundation. They are based entirely on superficial observation and attempt to "explain" facts by terms.

It is astonishing that serious authors accept as an explanation for protein action phrases like: "increase of the vitality of the living substance." This "explanation" is as perspicuous as the following, which we have culled from a medical publication: "peptone shock is proteinoform. . . ." Belfanti is justified when he considers such definitions as "a play on words rather than real facts."

The first serious attempt at an explanation of the mechanism of the action of various protein substances, can be traced as far back as 1909, to Nolf's study of peptone.

"The parenteral administration of foreign albumins gives rise to disturbances which are by no means specific, but can be produced to a higher or lesser degree by all foreign albumins; propeptone poisoning is the best studied example of this kind." This view is opposed to that of Widal. In 1917 Nolf made a great stride forward by establishing the most close analogies between the action of peptones and colloids. Moreover he demonstrated that neither colloids nor peptones exert a bactericidal action, but that the beneficial effect of this therapy is to be sought "in the disturbance of the protein composition or of the colloidal equilibrium."

In 1919 Nolf advanced the view that the ideas on the subject of serotherapy and vaccinotherapy prevailing at that time were too primitive, and that the effect produced by the injection of a serum or of a vaccine can be brought about by other agents than by the antibody introduced by or formed as a result of the injection.

In the same year Luithlen, in his attempt still further to penetrate into the mechanism of the action of foreign proteins on the blood colloids, discovered the modification of the permeability of the blood vessels, and assumed that this too is due to an irritation of the tissues.

In 1921 Widal adopted Nolf's conception and expressed the opinion that "the favorable reactions utilized by proteinotherapy are not of a chemical nature; they reflect the varying aspects of a conflict between the colloids." "The reaction provoked is always the same: shock." "The nature of the injected substances matters little; anything can be injected provided that it produces shock." Later this author attempted to establish a difference in the mode of action of these substances: The nature of the shock is in certain cases such as to "reëstablish a normal equilibrium," notably in diseases originating in "disturbed equilibrium or instability of the plasma." In other cases, for instance in infectious diseases, the favorable effect is attained by upsetting the equilibrium violently and suddenly; "it is treatment by shock." It is clear that Widal's differentiation of diseases into infectious and those caused by disequilibration is hardly exact, as will later be shown. In brief, all the theories of the older authors are, with the exception of Nolf's experimental, physiological work, merely beliefs, full of confusion, even of contradictions. The magic word "colloid" has fascinated the authors and is used as a panacea exactly as the antitoxins, lysines and *tutti quanti* were used in the past.

Let us then consider the work of Nolf, the orfly one of permanent value. It has culminated in establishing the identity of the actions of proteins, and (inorganic) colloids. To Nolf, protein therapy is colloid therapy. What is the foundation of this theory?

A systematic study of the *symptoms* will bring us nearer to the point. An important fact must be emphasized from the very beginning; the symptoms vary perceptibly according to the site of the injection. They are somewhat indistinct in hypodermic injections, more pronounced in intramuscular and attain their full violence in intravenous injections. Moreover there is an essential difference between the symptoms according to whether the colloids are introduced into the circulation or into the vertebral canal. In the latter case there appears besides the common symptoms, a very characteristic meningeal syndrome, Kernig's sign, rachialgia, headache, etc. (Lomonier).

Widal, Abrami, Lemièrre, describe the reaction following hypodermic injection of typhoid vaccine as follows: "Immediately after the injection there is the local reaction: redness, and a more or less marked sensitiveness around the site of the injection. Edematous swelling appears shortly after the injection, generally reaches its maximum in 10-12 hours and disappears in 36-48 hours."

The general reaction appears in two different forms: the one is "a reaction specific in nature; it is brought about by the poisons (*sic*) diffusing from the protoplasm of the injected bacteria." It is an abortive typhoid fever reduced to a slight indisposition, with headache, anorexia, lumbago, rise of temperature to 38°-39° C. It begins several hours after the injection and disappears after 24-36 hours.

In other cases the postvaccinal (typhoid) reaction may be more pronounced and extend over a period of several days.

"The other type of reaction, altogether exceptional, is the common albuminoid shock, which sets in very soon after the injection and is characterized by dyspnea, a tendency to lyopthymia, cyanosis, anuria, herpes; the symptoms may arise isolated or together."

The authors do not mention in connection with this latter type, the symptoms regularly observed by other clinicians (Delbet, Boulay and Winter, Hartmann, Auvrey, etc.), namely, rise of temperature, urticaria, chills, hot flashes and perspiration. If we add these symptoms to the above picture, the difference between the two reactions which these authors tried to establish, disappears entirely except for the intensity.

The reactions produced by the same vaccine under identical experimental conditions (dose, time and mode of injection) vary extremely according to numerous clinical observations, so as to make a group differentiation impossible. However, in spite of all these variations, this reaction can be easily recognized by the suddenness of its onset, the rise of temperature, chills, etc. . . . After having seen several of these typical reactions one is convinced that the differences are only in degree, not in kind.

What is the symptomatology of these serum reactions?

Belfanti reports two very interesting cases following the intravenous injection of specific serum and normal bovine serum in malignant pustula (*vide supra*).

(a) *Specific serum.* 15 minutes or at the latest 30 minutes (the time varies with the individual, the dose and the mode of introduction) after the intravenous injection of 40-60 cc. of the serum, violent chills occur, followed by a rise of temperature of 1°, 1.5° C. or more, and a few hours later a rapid

drop in temperature. Leucopenia ensues and finally a persistent leucocytosis.

(b) *Bovine serum.* The symptoms are the same as with specific serum. Sometimes the symptoms can attain "such a violence as to result in collapse and finally death from asphyxia, internal hemorrhage, probably capillary thrombosis, etc." We have, besides that, a detailed knowledge of the serum reactions and we have ourselves described a few highly alarming cases. N. Fiessinger reports a particularly impressive case following the injection of goat serum: The (hypodermic) injection was not yet completed when the patient (female) fell to the floor unconscious, in an attack of convulsions, with incontinence of urine, cyanosis, filiform, difficultly palpable pulse, and irregular respiration. The coma lasted for several hours, the collapse several days and ended in a rash of urticaria.

Let us compare this symptomatology with the reaction following peptone injection.

"The effect depends on the dose administered. . . . In case of a small dose (0.01 g. per kg. body weight) . . . a rise of temperature is observed one or two hours after the injection, and persists several hours. If the dose is somewhat larger, the same initial rise is occasionally associated with fairly pronounced chills. In case of strong reactions, the patient exhibits shortly after the injection strong heart palpitation, often also local arterial sounds in the lumbar region. The face is flushed, the patient complains of headache. The pulse is fast; above a certain frequency it becomes small and depressible. The respiratory rate is increased. Patients with pulmonary affections often begin to cough. The cough should be suppressed, for, if exaggerated, it is liable to be followed by nausea and vomiting.

"The attack lasts only a few minutes and leaves no effect if it is not pronounced. But more violent reactions may take place; the patient has an attack of dyspnea and respiratory anguish. The pulse is fast, 140-160. After some time a more or less extensive urticaria may appear on the trunk, the limbs and the face. . . . This is the anaphylactic shock syndrome. The more serious the condition of the patient the more readily this syndrome is established." (Nolf, pp. 93-94.)

This symptomatology furnishes the undubitable evidence that the reactions following the injection of vaccines—homo- or heterologous serums are identical with those of peptone shock. The same experience was made by Buchner already in 1890 with protein substances extracted from microbes; by Luedtke with deuteroalbumoses; by Weichardt in the course of his many investigations of all albuminoid substances.

R. Schmidt reports similar reactions in his first forty experiments with milk therapy: their intensity varied, but there was always a rise of temperature to 40° C., chills, etc.; Linding has elicited the same reaction by casein injections, E. F. Muller with whey, sodium nucleinate, etc. . . .

And arsenobenzene shock? * Are we not justified in classifying it among these phenomena? Is it not one of the most convincing examples of humoral shock?

On the other hand, all these reactions are identical with the reaction resulting from the intravenous or even intramuscular injection of "collobiases" and certain colloids of therapeutic value, as we have pointed out in the first part of this paper.

We are then necessarily led to the only obvious conclusion: *The symptomatology of the reactions following the introduction of any serum, vaccine,*

* See paper by H. M. Spencer in this volume. *J. A.*

protein substance and colloid is always the same: it is the symptomalogy of colloidal or humoral shock. Of this complex of symptoms let us consider the main two: (1) the fever and (2) the affection of the respiratory system. They are constant factors although certain observations point to the contrary. These contradictions are, however, superficial as will be revealed by a closer study.

Thus certain authors have observed that, in infectious diseases, the protein injection is followed by a rapid drop of temperature. Here again the dose, the site of the injection, as well as the nature of the proteins are of material influence. Popielski, Weichardt and Abderhalden have established the facts governing this relation.

(1) Genuine (not denatured) albumins in large and small doses produce on first and second injection a rise of temperature.

(2) Peptones and amino acids cause in small doses (4 g. for the dog) a slight augmentation of the fever, in large doses (15 g.) a very considerable rise of temperature.

(3) Histone and globine produce initially a drop of temperature followed after ten minutes by a slight rise.

(4) Protein substances purified by dialysis, raise the temperature when given in small doses, and in large doses cause a drop from the very beginning.

(5) Bacterial proteins are by far the most effective.

(6) Intravenous injection has a notably more violent effect than hypodermic.

It is therefore intelligible that the fever reaction varies from one case to the other according to the nature of the protein substances.

The injected dose is also of great importance as was demonstrated in 1918 by Hashimoto. In his experiments the temperature was lowered by small doses, but rose after the injection of a large dose of antigen.

Thus the first symptomatic factor always present in the reactions following protein therapy is *thermal alteration.*

The other factor is the invariably present *affection of respiration.*

We have already mentioned the observation of Nolf, according to which peptone shock elicits cough in patients with pulmonary lesions.

Longin and Camusset, Cuneo and Roland report the sudden outbreak of severe pulmonary edemata in patients suffering from lung congestion. The occurrence of pulmonary affections even in absence of preëxisting lesions is sufficiently confirmed by the symptomatology of moderate shock.

Avery, Chickering, Cole and Dochez have observed a typical attack of pneumonia after the first injection of antipneumococcus serum. The far reaching analogy between an attack of pneumonia and protein shock was also pointed out by Widal. On the other hand the rôle of the lungs in anaphylactic shock and colloid shock was sufficiently demonstrated by many authors, particularly by Foà and Aggazzotti.

These two factors can be considered as the two outstanding features of protein reaction. The varying aspects assumed by this reaction is caused solely by the variations of these factors; the constant elements are revealed by a systematic and exact investigation.

Our conclusion appears rather formal: *The symptomatology of the reactions following the introduction of various protein substances is that of colloidal, humoral shock.*

No great importance should, however, be attributed to this analogy of symptoms—as clinicians only too readily are inclined to do. In fact,

the problem would hardly present any interest if the analogy were to cease here.

We have already stated in the preceding part of this paper in connection with the differentiation of shocks, that the organism does not have at its disposal an infinite variety of symptoms by which to indicate the onset of anomalous conditions. But we have discovered other, far more interesting analogies.

Let us proceed in our investigation to the study of *humoral changes*. The most extensively studied of these phenomena is *leucopenia*.

Goldscheider and Jacob have shown that it is the result of a sudden change in the distribution of the blood in the vessels; that it is only peripheral and that an accumulation of leucocytes takes place in the capillaries of the lungs, the liver and the spleen. Naegeli wrote in 1912 about the "attracting action" of these capillaries. The leucopenia is followed by a persistent leucocytosis. This statement has been confirmed by a great number of authors, among whom are Weichardt, Zehnter, Mauriac, and by the clinical results of Widal, Maixner, Ducastello, Dziembowski, etc. Leucopenia with subsequent hyperleucocytosis was always found after the injection of vaccines, serums, colloids, peptone and various protein substances. *It is a common symptom of all shocks.*

The same holds for platelet deficiency according to the few results of Achard. The same is also true for the *drop of arterial pressure*. It was observed by Portmann after vaccine injection (1 cm. Hg. on the Pachon manometer) particularly for the minimum pressure. It is similarly associated with serum injections (Proszynski). It was described by Nolf as a result of rapid injection of peptone into dogs and clinically in patients with typhoid fever. *Weymeersch* has demonstrated that lowered pressure accompanies the injection of organ extracts. It was observed by Battelli and Mioni after the introduction of heterogenous serums.

According to Foà and Aggazzotti, colloid reactions are always attended by a decrease of arterial pressure, an observation confirmed by Cuneo and Roland. On the other hand a drop in arterial pressure is the most reliable indication of cell shock. Here is another perfect analogy: *arterial pressure is lowered in all shock reactions.*

The harmony is less perfect with regard to blood clotting. The existing data are contradictory, the experimental facts very scant. Besides the investigations of Hayem on the importance of the magnitude of the dose, and those already cited of Nolf on the effect of the rapidity of the injection, there are also the experiments of van der Velden, which, however, have not furnished conclusive results. The only phenomenon experimentally approached and established, is the incoagulability of the blood during cell shock. This fact was established by the remarkable work of Arthus, confirmed by the painstaking investigations of Modrakowski, and finally by Weymeersch in connection with the injections of organ extracts. In spite of this we read in a number of clinical publications that accelerated clotting is the outstanding symptom of cellular shock. In serum shock the blood remains liquid after 24 hours. Recently Quagliariello has reported the incoagulability of the blood of the dog after 24 hours following the injection of octopus blood. We have described the same phenomenon in the guinea-pig as the result of an injection of muraena (a kind of eel) blood. In a case of death following blood transfusion the blood was found on necropsy to be liquid (Kuczynski).

The observed acceleration of clotting has induced several clinicians to

devise a coagulation therapy with the aid of serums of sensitized animals (Dufour). It is, however, advisable to consider physiological results as the reliable foundation and to observe a certain caution with regard to clinical observations and therapeutic successes.

An agreement cannot be expected with regard to other alterations of the blood, which have been stated chiefly by clinicians—phenomena of which not only the mechanism but even the very existence is still a profound mystery. We are referring to the alterations of the "agglutinizing, bactericidal, phagocytic and opsonizing power," etc. . . . But as soon as we turn to experimental facts we find in every instance all these analogies in addition to new and closer ones. If we ever feel justified in forming an opinion, it is only on the foundation of these experimental tests. In 1917 Harris Boughton found lesions of the vascular tunica as a result of protein injections. Luithlen has stated only recently that serum reactions are associated with a decrease of vascular permeability. Thus the skin of an individual who has had a serum injection is less sensitive to the local irritation produced, *e.g.,* by croton oil. Starkenstein has observed the same phenomenon in the case of chrysarobine. Basing on this fact, Spiethof has succeeded in increasing the tolerance to arsenobenzene, and even in suppressing arsenobenzene shock. The same holds true for cellular shock (Starlinger). Quite recently Caspari has observed that in the preanaphylactic stage the sedimentation rate of the red blood cells is lower. Wiechmann and Schroder have stated an accelerated sedimentation in the moment of shock. The same phenomenon appears after the injection of various proteins (Linsenmaier, Löhr), of milk (Landsberg) and of caseosan (Rosenberg and Adelsperger). Another analogy was rediscovered by Miss Mendeléjeff: the change of pH in shock and following injections of foreign proteins such as milk, various emulsions of globules, is independent of the nature of these substances. This finding of Miss Mendeléjeff confirmed the old data of Segale regarding the decrease of pH in dogs in anaphylactic shock and in peptone shock. Quite recently Geleza has found a perfect analogy between the physico-chemical changes caused by serum injections and those described in contact shock and vaccine shock. He finds that a definite decrease of surface tension always appears suddenly in the early hours. A complex of physical changes was pointed out somewhat earlier by Rosenberg and Adelsperger. These authors have made a comparative experimental study of the reactions following the injection of distilled water, artificial serum, trypaflavine and caseosan, and have found a close resemblance between the actions of all these substances with regard to the faculty of fibrinogen and of the "globulins" for flocculation, the sedimentation velocity of the red cells and the surface tension. Only in the case of caseosan were flocculation and sedimentation accelerated. The surface tension decreased rapidly. Injections of distilled water, artificial serum and trypaflavine had no effect on these biological or physical characteristics.

The actual state of our knowledge makes it highly probable that *protein therapy, similarly to colloid therapy, is a therapy by shock.*

In the light of this knowledge, Widal's differentiation of therapy by shock, and a preventive or restoring protein and colloid therapy, appears altogether unjustified. The same substance cannot react in two different ways. The effects may vary according to the dose, and the condition prevailing in the organism in the moment of its introduction, but the mechanism is in both cases the same. And this is the only important point.

The *mechanism* of all the reactions following the injection of most different

vaccines, serums, bacteria, of various proteins, colloids, sometimes even of ionized substances, is identical. It is shock. *Protein therapy acts by shock.*

Does this conclusion intimate that the shocks provoked by all these substances are identical? That no specificity whatever of physical or chemical nature can be attributed to them?

Before answering this question we will carefully study the problem of blood transfusion as it affords valuable information.

III. Blood Transfusion

A review of the actual state of our knowledge of blood transfusion leaves us with the impression that the problem is a cruel and discouraging riddle. The experimental facts are of no avail. And yet only physical chemistry, the foundation of all experimental research of life phenomena, can give us the answer.

The following precautions have been established by modern research as indispensable in blood transfusion: Patient and donor must be not only of the same race, the same sex, the same age, the same diet, but even of the same family, and must have the same individual history, the same temperament, in other words of the same "humoral history." In view of all these limitations we are induced to ask whether transfusion is really a *transfusion, i.e.,* the introduction of an absolutely identical blood or rather an *infusion?* We shall see that the reactions accompanying all blood transfusions are particularly apt to support the latter interpretation. Let us begin our contention by disproving the legendary belief of the harmlessness of small blood doses. The cases of death caused by the intravenous injection of 3 cc. of blood (Cruchet) of 5 cc. (Schnell), of 120 cc. (Kuczynski) sufficiently demonstrate the possibility of irreparable accidents caused by very small doses.

An idle discussion was raised some time ago by authors insufficiently acquainted with the clinical literature, on the promptness with which accidents occur after transfusion. In animal experiments ultrarapid reactions were observed even after hypodermic injections by Jadassohn, Biberstein, Prausnitz and Kuester, Eskuchen and others. Cruchet reports a case of death immediately after the completion of an intravenous injection of 3 cc. blood. Kuczynski describes a fatal case one hour after transfusion. In the case of Pepper, death occurred seven days after transfusion. As to minor symptoms, some appear very promptly (fever, dyspnea, lumbar pain), while others become manifest only after a certain time (hematuria, rashes, albuminuria, headache, etc.).

Thus no rule can be established with regard to this point. Guillain's statement that reactions never appear after the second day must be abandoned.

What is the symptomatology of these symptoms, and how frequently do they occur?

Fever is the most common after-effect. It is always present after transfusions according to certain authors (Lewisohn, Wederhake, H. Fischer), etc. *Lumbar pain and urticaria were observed in about* 15 per cent, hematuria, albuminuria, vomiting and headache in about 10 per cent of cases. Finally there are about 50 cases of death in about one million of reported transfusions. (Landois.)

The passing symptoms can be summarized as follows: Lumbar or diffuse, radiating pain, chills, trembling, dyspnea, hot flashes or pallor of the face, profuse perspiration, rise of temperature, accelerated pulse; or lowered tem-

perature and filiform pulse, headache. These disturbances last from 15-30 minutes and leave no after-effect, except a passing fatigue. Some time later —on the next day or after a few days—urticaria, edema, renal disturbances and pains in the joints appear.

A description of the symptoms accompanying death after blood transfusion may be quoted from Cruchet: Anguish, pallor, violent headache, dizziness, dimness of vision, fast, filiform pulse, dyspnea, respiratory failure; ecchymosis over the entire body; immediate death. What is the nature of the anatomical lesions? Landois in 1875 and recently Kuczynski have recorded the lesions in a careful necropsy performed two hours after death. The blood is liquid. Embolism of the pulmonary capillaries, confirmed by microscopic examination; even obliterated capillaries in the deformed renal tubules. Hemorrhages in the liver; spleen enlarged.

Coca some time ago described the same anatomical lesions in rabbits following the injection of foreign blood. They are the well-known lesions of anaphylactic shock as they have been observed in animal experiments and 'in clinical cases. The symptoms attending death and the sequelae caused by transfusion including the alteration of temperature, are characteristic of contact shock. There is no doubt left in this regard. Blood transfusion presents itself to us as an infusion of blood, an injection of a foreign protein or a foreign colloid. The reaction which it provokes is unmistakably one of an immediate and profound disturbance of the colloidal blood equilibrium. *Blood transfusion is therapy by shock.* The so-called blood transfusion actually does not exist. Moreover, if we keep in mind that the red cells and all the transferred elements cease to exist rapidly under the new conditions—a fact known since the work of Bichat, Magendie and Brown-Séquard and corroborated by ultramicroscopic observations—the idea of rejuvenation and immortalization of individuals by transfusion, which obsessed the mind of man in the past centuries, can, to our present knowledge, be considered as disposed of.

IV. THE MECHANISM OF SHOCK

The study of the mechanism of shock is confounded with that of anaphylaxis. Richet's assumption of a purely chemical formation of an "anaphylactic" poison was superseded by the ferment theory of Friedberger. This hypothesis had to be abandoned after it has been demonstrated that the phenomena of shock can be produced in absence of nitrogenous bodies. The first proof was furnished by Kopaczewski and Muttermilch in 1914 and corroborated by Bordet and Zunz, Novy and de Kruif, P. S. Schmidt and others. At the same time we established a relation between the ultramicroscopic flocculation appearing in serums when brought into contact with suspensions or with colloidal gels and the cause of the shock phenomena. This statement was the starting point of our colloidal theory of shock. On June the 30th, 1914, cinematograph records obtained ħ' collaboration with Commandon were presented before the Société de Biologie de Paris. Later, in 1917 it was definitely established in collaboration with Behm and Dahl that the relation between flocculation and shock is one between cause and effect.

Starting from this hypothetical basis we have undertaken a complete series of experimental verifications in order to put the hypothesis to the test.

In 1916 we succeeded in suppressing the shock produced by an injection of murene blood, by lowering the surface tension and increasing the viscosity

of the blood. In 1918 we suppressed, by the same means, the shock following the injection of serum which had been in contact with gels.

In 1919 we obtained, in collaboration with Vahram, the same result in anaphylactic shock.

These results have been confirmed by Pesci and Lumière for sodium oleate, by Lhermitte, Duhot, Arloing, and Siccard for bicarbonates, carbonates and sugars. In 1918 we demonstrated the diminution of surface tension and the inversion of the electric charge of the serum "globulins" following the shock. These results have been confirmed by Zunz and are in keeping with the older findings of Thiele and Embleton.

At the same time we have shown, in collaboration with Gruzewska, the importance of the electric charge in the production of humoral shock.

In 1920, in collaboration with Roffo, the importance of the central nervous system in shock was disproved. All these findings have enabled us to formulate a physical theory of shock. *The introduction of foreign substances of colloidal nature into the circulatory system effects immediate disruption of the micellar equilibrium resulting in flocculation. This flocculation begins in vitro and continues in vivo causing, through obstruction of the capillary vessels, asphyxia with all its usual consequences.*

This interpretation applies in the first place to the shock produced in guinea pigs by serums, previously brought into contact with suspensions and gels, and to anaphylactic shock in the guinea-pig. The conditions determining shock in other animals, as well as the mechanism of shock brought about by other means, may be different. Indeed, a condensed analysis of the facts reveals the existence of actual differences in contrast to the view of certain authors (Lumière) that all shocks are produced by micellar flocculation.*

The conception of colloids has to such a degree fascinated authors not sufficiently familiar with this state of matter, that they attribute to flocculation the rôle of a Deus ex machina (death and disease are determined by flocculation).

Calm reasoning, however, enables us to understand that there are essential differences between the shocks. To begin with anaphylactic shock must be considered separately. It occurs only after a certain period of labilization: apparently the intervention of the cells is necessary to bring it about. On the contrary the other shocks follow immediately upon the introduction of foreign colloids. The first shock can be termed anaphylactic or *cellular shock;* the other in contradistinction to it, *humoral shock.* The origin of the various humoral shocks can be different, regardless of the analogy of symptoms. For instance the introduction of a substance with a very low surface tension, will necessarily produce intravascular lysis, and the asphyxia resulting from it will furnish the analogy of symptoms. The injection of a suspension will immediately result in intravascular coagulation. Again asphyxia with analogous symptoms is provoked. It is very important to know these differences, not only from the theoretical but also from the medical point of view, since the medication should be appropriate to the kind of shock.

This colloidal theory has found a large number of followers, and has stimulated numerous researches which in a general way have served to support it.

Thus on the subject of *flocculation* an entire series of facts has been established which indirectly confirm our theory. In 1920 Dold has nephelometrically confirmed the presence of flocculated micells in serums which have been in

* See paper by A. Lumière, and also that of E. Zunz, both in this volume. *J. A.*

contact with microbes or gelose. This fact was stated by ourselves already in 1914 and was later, in 1917, studied ultramicroscopically in all its details in collaboration with Dahl and Behm. Very recently Dale and Kellaway have confirmed the presence of micellar agglomerates and Gregorio y Turio made the same observation when mixing, according to our procedure, serum of a labilized guinea pig with the sensitizing serum. Moreover, by drawing serum from a guinea pig sensitized to antidiphtheric serum after a chloroform-water injection, and mixing the serum with antidiphtheric serum, Gregorio y Turio could detect no flocculation. The importance of flocculation in humoral shock was also proved by ultrafiltration experiments. Schmidt has made serums, altered by contact with gelose or microbes, perfectly harmless by filtration through a filter candle. Moreschi and Golgi have obtained the same result as early as 1913. Dale and Kellaway have confirmed the experiences of Moreschi, Golgi and Schmidt.

In several of the older publications Friedberger maintains his old opinion and tries to prove that filtration has no significance. He particularly points out that the action of the filter is not purely mechanical, but is largely determined by adsorption phenomena and that it is ineffective as soon as adsorption is eliminated. ("Berkefeld candles prevent the poison from passing, only as far as adsorption enters into consideration." . . .) There is nothing new in the statement that adsorption plays an important part in ultrafiltration. On the other hand the fact pointed out by Friedberger that the effect produced by filtration varies with the nature of the filter, is not surprising, since adsorption depends on the nature of the filter. The important fact is only the incidental disappearance of micellar agglomerates and of the harmful properties of the serum, by the simple process of filtration. The mechanism of the disappearance, whether it be mechanical absorption, adsorption or chemical reaction, has little significance from this point of view. This correlation was found in our experiments (published with Dahl and Behm) on the prevention of shock by soaps and anesthetics, and was confirmed by Gregorio y Turio. Friedberger's experiments evade the main point; in his last publication he tends to consider the guinea pig as a chemical reagent. He speaks of definite doses which produce the irremediable kinds of shock. Even an exact determination of "anaphylatoxin" is undertaken. Is this really possible, even in the hands of a worker as experienced as Friedberger?

Our conception of *the effect of flocculation* is similarly confirmed by several publications. We have advanced the hypothesis that blood clots act as centers of attraction for cell agglomerations. The latter obstruct the pulmonary capillaries as soon as they reach their level, and cause death by sudden asphyxiation. This explains the mode of action of vasodilators. The attempt was made to oppose our view by the assumption of a reflex vasodilatation due to the excitation of nerves, vessels and the central nervous system. We shall see later what part in the production of shock has to be attributed to the nervous system. At present we shall proceed to demonstrate with the aid of various publications the correctness of our assumption. Manvaring, French and Boy are led by their detailed researches to the conclusions that shock is accompanied by (1) vasoconstriction of the pulmonary and hepatic vessels; (2) vasodilatation in the other organs.

The same facts were also found by Kochmann and Schmidt. By introducing serums consecutively into different vascular regions and thus eliminating definite organs Forssmann arrives at the conclusion that asphyxiation through bronchial spasm is the cause of death in anaphylactic shock, as well

as in the Forssmann phenomenon. ("Finally only the bronchia and the arteriae coronariae cordis were left. Only the bronchia or the heart can be the site of the shock.")

Coca has studied the perfusion of the lungs of the guinea pig with physiological salt solution. His experiments proved that after anaphylactic shock, the pressure necessary to effect perfusion was ten times higher than in normal animals. The above statements apply to guinea pigs. In other animals the last stage of the shock may take place in another organ. According to Manwaring, Voegtlein and Bernheim, in the dog this organ is the liver. The same applies to the cat, the oppossum and all carnivorous animals, while in herbivorous animals the heart is usually affected. Liver, as well as heart, exhibit strong hyperemia, and the drop in arterial pressure becomes exceedingly severe according to the publications of Mautner and Pick.

The researches of Abelous and Soula on urohypotensine and those of Dale, Abel, Kubota and Beresin on histamine, can also be cited as evidence of pulmonary vasoconstriction and vasoobliteration in flocculation shock. The dominant rôle of the lungs in these phenomena is further demonstrated by the papers of Grossmann, Waele and Cesaris Demel. The objection has been raised that the antishock action of adrenaline is difficult to explain in conformity with this theory. We can, however, state that this action has been observed and asserted only by Milian and only in clinical cases; it was never experimentally confirmed. According to our own experience, the effect of adrenaline in clinical cases is sometimes nil and sometimes very severe. But even if the alleged preventive action of adrenaline in clinical cases were proved, it can be explained by pulmonary vasodilatation. This fact, unknown to Milian, was experimentally established by recent investigations, particularly those of Beresin, Snyder, Martin, etc. These authors have moreover stated that the effect of adrenaline depends on the pH of the blood. Since the pII suffers an alteration in shock, the action of adrenaline and possibly of many other substances will find its experimental explanation. The action of adrenaline therefore does not constitute an argument against our conception of flocculation and obstruction of pulmonary capillaries, as Milian attempted to interpret it.

We come now to the rôle of the nervous system in the phenomena of flocculation shock.

Our experiments on the prevention of anaphylactic shock by general anesthesia have led us to the conclusion that the central nervous system has no influence on the production of shock phenomena. The results of these experiments were not considered to be sufficient proof by certain authors, who tried to interpret them as a result of irritation of the nervous system. Our theory was, however, confirmed in a striking manner by the elegant experiment of Daussain. This author has subjected guinea-pigs to electric anesthesia as described by Stéphane Leduc, and has shown that this kind of anesthesia affords no protection against the anaphylactic manifestations. The view of the dominant rôle of the central nervous system in anaphylactic shock, up to the present time considered as an experimental fact, has to be discarded.

Is the same true of the autonomous system? Do the disturbances of the vagus-sympathicus play an important part in the production of shocks or even of all shocks apart from the phenomena of chemical intoxication, which were confused by these authors with colloid reaction? This question, although worth studying, is not of such an outstanding interest as the preceding. The disturbances of the nervous system are, and always will be, incapable of

furnishing an explanation for the physical alterations attending shock: micellar agglomeration, lowering of surface tension, inversion of the electric charge of the "globulins" (Kopaczewski), increase of the viscosity of the blood without correlated increase of the plasma viscosity (Zunz), increase of the refractive index (Segale and Zunz), depression of the cryoscopic point (Segale, Zunz), and a good many others.

The differentiation of shock conditions, which we have established in spite of the hasty generalization of certain authors, has been confirmed and more exactly determined by recent researches. We have observed with Zunz that the effect produced *in vivo* in the mesentery of the frog, varied with the nature of the substance introduced into the circulation. Soaps caused hemolysis, while colloidal iron, similarly to animal carbon, gave rise to intravascular coagulation. The experiments of Schepfer (unpublished) establish the fact that lecithins, when injected, actually form a film around the blood corpuscles, thus preventing gas exchange. The result is rapid asphyxia. Schmidt found in harmony with Kopaczewski and Commandon (1922) and at variance with Lumière, that animals which have survived anaphylactic shock are by no means immune against other shocks, e.g., lytic or thromboplastic. Dale and Kellaway state substantial differences between anaphylactic shock on one hand, and shock caused by gelose or starch on the other. The problem of differentiation also seems to be solved in the sense which we proposed since 1920.

Let us finally discuss several papers relative to *flocculation*. The flocculation theory of shock is supported by the therapeutic experiences reported by G. Scholz on the beneficial effect of camphorated oil in arsenobenzene shock. It is equally supported by the investigations of Robert Duhot, which confirm our results on the prevention of arsenobenzene shock by saturated glucose solutions. But the most interesting evidence in favor of the flocculation theory, we find in the experiments of Kritchewsky. This author has shown that the extract of the plant *Cotyledon Scheidekeri* has the faculty of flocculating serum and bacterial extracts as well as of agglutinizing bacteria, red blood corpuscles and laked blood. He has injected definite amounts of this substance into guinea pigs and always succeeded in causing death with the symptoms and anatomical lesions characteristic of anaphylactic shock. He has reproduced these phenomena in the rabbit, and has observed the phenomenon of Arthus as a result of subcutaneous injections. Kritchewsky arrives at the conclusion that anaphylactic shock is due to a change in the dispersity of the plasma colloids of the blood. Dale reaches the same conclusion although his terminology is different: "We are entitled, I think, to suppose that, when antigen needs antibody in the specifically sensitive cells, the immediate effect is a change in the state of dispersion of the colloids entering into its structure." The Italian anatomo-pathologist Cesaris Demel believes that the sudden humoral and anatomo-pathological modifications of anaphylactic shock can be explained only by flocculation, a phenomenon reversible in its first stage (complex reversible colloids).

In opposition to Friedberger's contention based on anaphylactic phenomena observed outside of the body fluids on isolated organs, C. Demel demonstrates in his experiments on anaphylaxis of the isolated heart, which he was the first to carry out in 1910, that these phenomena can be satisfactorily explained by the flocculation theory. Demel refers particularly to the rapid appearance and disappearance of granules in the cell protoplasm in albuminoid degenera-

tion; to the necrosis which appears as a gelation of protoplasm preceding the sol state. He emphasizes the fact that this necrosis sometimes represents the final reaction in local anaphylaxis.

According to Cesaris Demel the anatomical alterations constitute a strong argument in favor of the flocculation theory: "The theory finds a valuable support in the interpretation of the anatomical alterations, since it permits us to outline the anatomical manifestation in a simple, comprehensive and convincing manner. By assuming that flocculation is the cause of vascular obstruction, we are led to understand why anaphylactic manifestations can be different in different species of animals. Sometimes the symptoms and lesions appear in the respiratory, sometimes in the digestive system, in other cases on the skin. This, no doubt, bears relation to the different extent of vascular distribution in the different organs, as well as to the fact that in each animal species certain organs have a congenital higher sensitiveness to vascular obstruction." We have cited Demel at length because objections have been raised with regard to the obstruction of pulmonary vessels. (Widal.)

Other experimenters, for instance the Italian bacteriologist Belfanti, consider the flocculation theory to be the most plausible one, in our present state of knowledge. In France, d'Hérelle considers flocculation as an "indubitable fact," but at the same time continues to invoke "antibodies and opsonins." He even sees no incompatibility between the importance of hydrogen ion concentration and the large group of imaginary substances.

Among German investigators, Doerr believes that the final humoral reaction is nothing else but a superacute intoxication, although he admits that adsorption and flocculation phenomena, etc., can play a certain rôle in sensitization: "Everything tends to indicate that the highly reversible physical reactions, such as adsorption and flocculation without any chemical change of the reacting component, participate in the precipitations of immunization or at least in their initial stage, which alone is of importance in anaphylaxis. The conception of a primary physical, but humoral reaction is nothing but a detour and eventually leads to the assumption of a relation between the induced toxicity of the blood plasma (due to substances inhibiting adsorption, to the activation of coagulating ferments, to disturbances of the colloidal equilibrium) and the physical changes of the blood. This brings us back to the conception of acute intoxication."

It is clear that the distinguished Swiss scientist overlooks the difference between physical and chemical reactions. His theory of "adsorption of pre-existing toxic substances or activation of coagulating ferments" may be correct in their principle, but his ideas of the theory of flocculation are decidedly erroneous.

A summary of this general review leaves with us the impression that the physical theory of contact shock is proved in every respect, and that the number of experimental investigators adhering to this theory is growing. We believe we have demonstrated the great importance of colloids for therapy and thrown some light on this confused chapter of pharmacodynamics.

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APPENDIX

•The Explanation of the Colloidal Behavior of Proteins [1]

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I

This year's Pasteur lecture coincides with the commemoration of the hundredth anniversary of Pasteur's birth. The application of •Pasteur's ideas and discoveries has benefited humanity to such an extent that they have become part of the consciousness of civilized mankind. What is, perhaps, less widely understood is the fact that Pasteur changed the method of medical research. In the study of infectious diseases Pasteur substituted for the method of hit or miss (with the chances infinitely in favor of missing) the method of a definitely oriented search which never fails to give results when properly applied. Thousands of physicians had studied infectious diseases before Pasteur, but they tried to solve their problem by starting from observations of the symptoms of some special disease. This led to no result for the simple reason that without knowing beforehand for what to look—or, in other words, without knowing the general cause of infectious diseases—it was impossible to discover the cause of any special infectious process. Pasteur reversed this method by his discovery of the action and omnipresence of microorganisms, leaving it to the medical men to look for the special agency in the individual cases.

There is little doubt that the old empiricism, still in vogue in some other fields of medicine and in the physiological sciences, must be replaced by the more rationalistic method of Pasteur of knowing the general fundamental principles before attempting to explain the more special phenomena, since, unless we follow this method, we never know which of the details we observe are significant and which are negligible.

II

Living matter is essentially colloidal in character and we can not well conceive of an organism consisting exclusively of crystalloidal matter. This fact

suggests that life phenomena depend upon or are intrinsically linked with certain characteristics of colloidal behavior. It is, therefore, natural that a systematic study of the nature of special life phenomena should be preceded by a scientific theory of colloidal behavior. By a scientific theory, however, we do not understand speculations or guesses built on qualitative experiments or no experiments at all, but the derivation of the results from a rationalistic, mathematical formula which permits us to calculate, with an adequate degree of accuracy, the quantitative measurements of colloidal behavior.

Proteins are amphoteric electrolytes which are capable of forming salts with either alkalis or acids. With alkalis they form salts like Na proteinate, Ca proteinate, etc., and with acids they form salts like protein chloride, protein sulfate, etc. Whether they do the one or the other depends on the hydrogen ion concentration of the protein solution. There is one definite hydrogen ion concentration at which a protein can combine practically with neither acid nor alkali, and this hydrogen ion concentration, which may be different for different proteins, is called the isoelectric point. The isoelectric point is (in terms of Sörensen's logarithmic symbol) for gelatin and casein at pH 4.7; for crystalline egg albumin at pH 4.8. Gelatin can combine with acid only or practically only when the pH is less than 4.7 and with alkali only or practically only when the pH is higher than 4.7. Or in other words, when a salt, e.g., NiC₂ is added to gelatin solutions of different pH, Ni gelatinate can only be formed when the pH is greater than 4.7; and when K₄Fe (CN)₆ is added gelatin-Fe (CN)₆ can only be formed when the pH is less than 4.7. This can be shown by methods discussed in a recent book.[2]

FIG. 1.—Titration curves of crystalline egg albumin.

The proof that proteins combine stoichiometrically with acids and alkalis can be furnished by titration curves. For this purpose (and perhaps for work with proteins in general) it is necessary to use as standard material protein of the pH of the isoelectric point. We have seen that proteins combine with acids only at a pH below that of the isoelectric point, which for gelatin or casein is about pH 4.7 and for crystalline egg albumin 4.8. It happens that at a pH below 4.7 most of the weak dibasic and tribasic acids dissociate as monobasic acids. Thus H_3PO_4 dissociates into H^+ and the monovalent anion $H_2PO_4^-$. Hence if acids combine stoichiometrically with isoelectric protein, it should require exactly three times as many cc. of 0.1 N H_3PO_4 to bring a 1 per cent solution of an isoelectric protein, e.g., gelatin or crystalline egg albumin or casein, to the same hydrogen ion concentration, e.g., pH 3.0, as it requires of 0.1 N HCl or HNO_3. Titration experiments show that this is the case. Furthermore, since H_2SO_4 is a strong acid, splitting off both hydrogen ions even at a pH below 4.7, the same number of cc. of 0.1 N H_2SO_4 as of HCl should be required to bring 1 gm. of isoelectric protein in
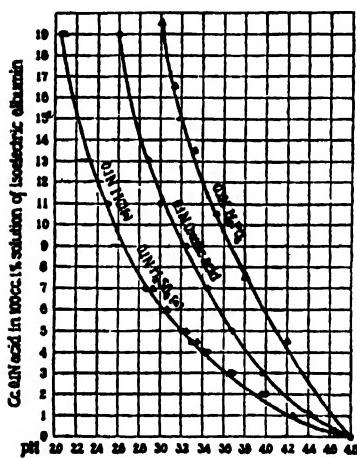
[2] Loeb, J., "Proteins and the Theory of Colloidal Behavior," New York and London, 1922.

100 cc. of solution to the same pH, e.g., 3.0, and this was found also to be true.

Figure 1 gives the titration curves for crystalline egg albumin for four acids, HCl, H_2SO_4, H_3PO_4, and oxalic acid. One gram of isoelectric albumin was in 100 cc. H_2O containing various cc. of 0.1 N acid. These cc. of 0.1 N acid in 100 cc. solution are the ordinates of the curves in Figure 1. The abscissæ are the pH to which the protein solution was brought by the addition of acid. It takes always exactly three times as many cc. of 0.1 N H_3PO_4 as it takes cc. of 0.1 N HCl or H_2SO_4 to bring 1 gm. of isoelectric albumin in 100 cc. solution to the same pH. In order to bring the 1 per cent solution of originally isoelectric albumin to pH 3.2, 5 cc. of 0.1 N HCl or H_2SO_4 and 15 cc. of 0.1 N H_3PO_4 must be contained in 100 cc. of the solution. To bring the albumin to pH 3.4, 4 cc. of 0.1 N HCl or H_2SO_4 and 12 cc. of 0.1 N H_3PO_4 must be contained in the solution, and so on.

Oxalic acid is, according to Hildebrand, a monobasic acid at a pH of 3.0 or below, but begins to split off the second hydrogen ion in increasing proportion above pH 3.0. The titration curves show that about twice as many cc. of 0.1 N oxalic acid as 0.1 N HCl are required to bring the 1 per cent solution of isoelectric albumin to the same pH below 3.0, while it takes less than twice as many cc. of 0.1 N oxalic acid as 0.1 N HCl to bring the albumin solution to the same pH if the pH is above 3.0.

It can be shown in the same way with the aid of titration curves that isoelectric albumin combines with alkalis in the same stoichiometrical way as any acid, e.g., acetic acid, would combine with the same alkalis. If the cc. of 0.1 N KOH, NaOH, $Ca(OH)_2$, or $Ba(OH)_2$ in 100 cc. solution required to bring a 1 per cent solution of isoelectric protein to the same pH are plotted as ordinates over the pH of the protein solution as abscissæ, it is found that the values for all four alkalis fall on one curve as they should if the combination occurred strictly stoichiometrically.

The same stoichiometrical results were obtained also with casein and gelatin by the writer, and with edestin and serum globulin by Hitchcock. There is little doubt that they will be obtained in the case of all proteins. It follows from this that proteins react with acids and alkalis in the same way as do amphoteric crystalloids like amino-acids. If the methods for measuring the hydrogen ion concentrations of protein solutions had been employed by the colloid chemists nobody would have thought of suggesting that proteins react with acids and alkalis according to the empirical adsorption formula of Freundlich instead of stoichiometrically.

The purely chemical character of the combination of proteins with hydrochloric acid can also be demonstrated by measuring the chlorine ion concentration of the solutions of protein chloride. When HCl is added to NH_3 (according to Werner) the H ions of the HCl are attracted to the nitrogen of the ammonia, while the Cl ions remain unaltered. The same type of reaction occurs when HCl is added to a solution of isoelectric gelatin. This was proven by measurements of the pCl of solutions of gelatin chloride. Different cc. of 0.1 N HCl were contained in 100 cc. of 1 per cent solutions of originally isoelectric gelatin and the pH and pCl of the solutions were measured, the pH with the hydrogen electrode and the pCl with the calomel electrode. It was found that the pCl was the same as if no gelatin had been present while the pH was, of course, higher; thus showing that part of the hydrogen combines with the NH_2 and NH groups of the protein molecule while the Cl remains free

(Table 1). Dr. Hitchcock has obtained similar results with crystalline egg· albumin, edestin, casein, and serum globulin, by using a silver chloride electrode, so that it is possible to state that these results are true for most if not all proteins.

TABLE I.

Cubic Centimeters of 0.1 N HCl in 100 cc. Solution	Solution Containing No Gelatin		Solution Containing 1 gm. of Isoelectric Gelatin in 100 cc.	
	pH	pCl	pH	pCl
2	2.72	2.72	4.2	2.68
3	2.52	2.54	4.0	2.53
4	2.41	2.39	...	...
5	2.31	2.29	3.60	2.33
6	2.24	2.26	3.41	2.25
7	2.16	2.18	3.23	2.18
8	2.11	2.12	3.07	2.11
10	2.01	2.01	2.78	2.025
15	1.85	1.85	2.30	1.845
20	1.72	1.76	2.06	1.76
30	1.55	1.59	1.78	1.60
40	1.43	1.47	1.61	1.47

The titration curves prove another fact, namely, that the salts of proteins are strongly hydrolyzed. When we add acid, e.g., HCl, to isoelectric protein, part of the acid combines with the protein giving rise to protein chloride, while the rest of the acid remains free. There is then an equilibrium between free HCl, protein chloride, and non-ionogenic (or isoelectric) protein. The more acid is added to originally isoelectric protein, the more protein chloride is formed until finally all the protein exists in the form of protein chloride. It is possible to find out from the pH measurements how much of the acid added is free and by deducting this value we know how much is in combination with the protein. By saturating the protein with acid the combining weight of a protein with acid can be found. Hitchcock found in this way that the combining weight of gelatin is about 1090.

III

The colloidal behavior of proteins shows itself in a peculiar effect of electrolytes—acids, alkalis or salts—on such properties as the swelling of gels or the osmotic pressure or viscosity of protein solutions. All these properties, swelling, osmotic pressure, viscosity, are affected by electrolytes in a very similar way; suggesting that all are due to the same cause. We shall see that by giving the explanation for one of these properties, osmotic pressure, we shall by implication give the explanation for all of them.

Measurements of the osmotic pressure of solutions of a protein—gelatin, crystalline egg albumin, casein and edestin—were made with solutions containing 1 gm. dry weight of originally isoelectric protein in 100 cc. of solution; and the 100 cc. of solution included also varying concentrations of 0.1 N acid. These solutions were put into collodion bags suspended in water free from protein. The outside water was at the beginning of the experiment

brought to the same pH as that of the protein solution, using always the same acid as that added to the protein. The measurements of the osmotic pressure were read after 18 hours when osmotic equilibrium was established. It was found that the osmotic pressure varied in a characteristic way with the pH of the protein solution and the valency of the anion of the acid used. This effect is shown in the curves in Figure 2 which were obtained from gelatin solutions. But the curves are similar in the case of other proteins such as crystalline egg albumin, casein or edestin. These curves show that the osmotic pressure of a protein solution is a minimum at the isoelectric point, that it increases when little acid is added until a maximum is reached, and that on the further addition of acid the osmotic pressure is again diminished. They show, moreover, that only the valency and not the nature of the anion of the acid influences the osmotic pressure of a protein solution. We know from the titration curves that in the case of H_3PO_4 the anion in combination with the protein is not the trivalent PO_4 but the monovalent H_2PO_4; and the curves in Figure 2 show that the influence of phosphoric acid and hydrochloric acid on the osmotic pressure is the same if measured for the same pH of the protein solution. Oxalic acid is a monobasic acid below pH 3.0 and we notice that the descending branch of the oxalic acid curve below pH 3.0 practically coincides with the descending branch of the HCl curve. The curve for the influence of H_2SO_4 is only about half as high as that for HCl and we know from the titration curves that the anion of protein sulfate is bivalent. It was found that all monobasic acids, e.g., HBr, HNO_3, acetic acid, etc., and all weak dibasic or tribasic acids, e.g., tartaric, malic, citric, etc., which below pH 4.7 dissociate as monobasic acids, give osmotic pressure curves identical with those for HCl and H_3PO_4. We may, therefore, draw the conclusion that only the valency but not the nature of the acid influences the osmotic pressure of protein solutions, that all acids which are monobasic on the acid side of the isoelectric point of a protein influence its osmotic pressure in the same way, and that this influence is considerably greater than the influence of strong dibasic acids like H_2SO_4.

If alkali is added to a solution of isoelectric protein it can be shown that the addition of little alkali increases the osmotic pressure until a maximum is reached when the addition of more alkali depresses the osmotic pressure again. All alkalis with monobasic cation like Li Na, K, NH_4, have the same effect at the same pH, while alkalis and all dibasic cations like Ca or Ba act alike, the curve for the effect of the alkalis with divalent cation being only about half as high as that of the alkalis with monovalent cation.

A third fact (discovered by R. S. Lillie) is that the addition of salts to a solution of a protein salt always depresses the osmotic pressure.

The curves representing the influence of acids and salts on the osmotic pressure are almost identical or very similar to those representing the influence of the same acids and salts on swelling and viscosity. These results are specific for colloidal behavior and any theory of colloidal behavior will have to give not only a qualitative but a quantitative theory of these curves.

It was suggested by Zsigmondy that the influence of acid on osmotic pressure was due to an influence on the degree of dispersion of the protein in solution, but since the degree of dispersion can not be accurately measured, this suggestion is only a qualitative speculation. But it is of no use even as a qualitative speculation since it fails to account for the fact that viscosity and swelling are affected in a similar way as osmotic pressure. The

correct explanation is as follows: When acid (or alkali) is added to a solution of an isoelectric protein, part or all of this is transformed into an ionizable protein salt according to the amount of acid added. This ionization of the protein causes the colloidal behavior on account of the inability of protein ions to diffuse through membranes which are easily permeable to crystalloidal ions, such as collodion or parchment membranes or the walls of capillaries or probably of all cells. Now it was shown by Donnan that when ever the diffusion of one type of ions such as colloidal ions is prevented by a membrane which is readily permeable to crystalloidal ions, an unequal distribution of the diffusible crystalloidal ions results on the opposite sides of the membrane. This unequal distribution of diffusible crystalloidal ions is the cause of the colloidal behavior of proteins.

IV

When a collodion bag is filled with a solution of gelatin chloride of pH 3.0 and the bag is immersed in an aqueous solution of HCl also of pH 3.0 but free from protein, acid is driven from the protein solution into the outside aqueous solution free from protein. Donnan has shown thermodynamically that when osmotic equilibrium is established the products of the concentrations of each pair of oppositely charged diffusible ions (e.g., H and Cl in the case of gelatin chloride) are equal on the opposite sides of the membrane. Let x be the molar concentration of the H and Cl ions on the outside, y the molar concentration of the free H and Cl ions inside the protein solution, and z the concentration of the Cl ions in combination with the protein; then equilibrium is defined by the following equation, first used by Procter and Wilson to explain the influence of acid on swelling,
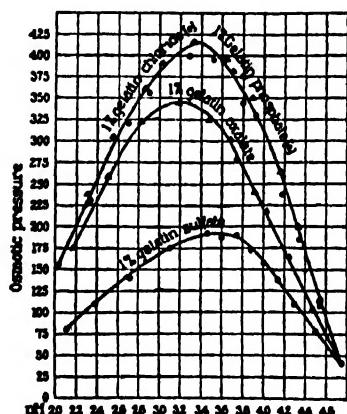
Fig. 2.—Variations in osmotic pressure in gelatin solutions.

$$x^2 = y(y + z) \qquad (1)$$

The first step in an attempt to explain the influence of acids, alkalis and salts on the osmotic pressure of protein solutions is to find out whether the variations in osmotic pressure under the influence of acids as shown in Figure 2 are accompanied by corresponding differences in the concentration of diffusible ions inside and outside the protein solution and whether these differences can be calculated from Donnan's equilibrium equation (1).

The writer was able to show that this is true by making measurements of a property of protein solutions, which had received little if any attention in colloid chemistry, namely, the measurements of the membrane potentials existing between a protein solution and the surrounding aqueous solution at the time of osmotic equilibrium.

Donnan's equilibrium formula can be written in the form

$$\frac{x}{y} = \frac{y + z}{x}$$

where $\dfrac{x}{y}$ is the ratio of the molar concentration of the hydrogen ions outside to the concentration of the hydrogen ions inside, while $\dfrac{y+z}{x}$ is the ratio of the molar concentration of the chlorine ions inside to that outside. Donnan had shown that there should exist a potential difference between the inside and outside solutions, which at 24° C. should be equal to $59 \times \log \dfrac{x}{y}$ millivolts or $59 \times \log \dfrac{y+z}{x}$ millivolts. Since pH inside is $= -\log y$ and pH outside is $= -\log x$, $\log \dfrac{x}{y}$ is equal to pH inside minus pH outside. pH inside and pH outside can be determined directly with the aid of the hydrogen electrode; $\log \dfrac{y+z}{x}$ is equal to pCl outside minus pCl inside and this quantity can be measured directly by titration or with the silver chloride electrode.

On the other hand, the P.D. between the protein solution and the surrounding aqueous solution across a collodion membrane can be measured directly with the aid of a Compton electrometer and a pair of identical indifferent calomel electrodes (and saturated KCl). If the unequal distribution of diffusible crystalloidal ions (e.g., H and Cl in the case of gelatin chloride) on the opposite sides of the membrane is really determined by the Donnan equilibrium, then the P.D. observed directly with a pair of identical calomel electrodes should be equal to the P.D. calculated in millivolts from the values $59 \times$ (pH inside minus pH outside) or from $59 \times$ (pCl outside minus pCl inside), where pCl or pH may be obtained by titration or by the silver chloride or hydrogen electrodes respectively. The writer has made these measurements and found that when various quantities of acid are added to solutions of isoelectric protein—e.g., crystalline egg albumin, or gelatin, or casein—the observed membrane potentials always agree with the membrane potentials calculated on the basis of Donnan's equation within one or two millivolts, i.e., within the limits of accuracy of the measurements.

The net result of extensive measurements of membrane potentials was, first, that when a protein solution, enclosed in a collodion bag (impermeable to protein ions but permeable to crystalloidal ions), is in osmotic equilibrium with an outside aqueous solution, the concentrations of crystalloidal ions in the protein solution and in the outside aqueous solution are not the same: and second, that the difference in the two concentrations can be calculated from Donnan's equilibrium equation.

V

We are now in a position to explain the osmotic pressure curves in Figure 2. The colloid chemists would have taken it for granted that such curves were due to an influence of the acids on the state of dispersion or on some other real or imaginary colloidal property of proteins. Before we have a right to indulge in such speculations we must realize that these curves of observed osmotic pressure are not exclusively the expression of the osmotic pressure due to the protein particles, or protein molecules, and protein ions alone, but are also the result of the demonstrable unequal concentrations of

the crystalloidal ions on the opposite sides of the membrane, caused by the establishment of a Donnan equilibrium. In other words, the observed osmotic pressure of a protein solution needs a correction due to the Donnan equilibrium before we can begin to speculate on the cause of the influence of acid on these curves, and it is our purpose to calculate the value of this correction.

We begin with the curve expressing the influence of HCl on the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin and we consider the distribution of ions inside the protein solution and in the aqueous solution outside the collodion bag containing the protein solution at osmotic equilibrium. We also assume complete electrolytic dissociation of gelatin chloride as well as HCl. Let a be the molar concentration of the protein molecules and ions, let z be the molar concentration of the Cl ions in combination with the ionized protein, let y be the molar concentration of the hydrogen ions of the free HCl inside the protein solution; the molar concentration of the Cl ions of this HCl is also y. In that case the osmotic pressure of the protein solution is determined by

$$a + 2y + z$$

From this must be deducted the osmotic pressure of the HCl of the outside aqueous solution. If x is the molar concentration of the H ions of the outside solution, it is also the molar concentration of the Cl ions. Hence the observed osmotic pressure of a protein solution is determined by the following molar concentration,

$$a + 2y + z - 2x$$

Figure 2 shows how this value varies with the pH of the protein solution (i.e., y). In order to arrive at a theory concerning the influence of HCl on the osmotic pressure of protein solutions it is necessary to calculate the value of $2y + z - 2x$ and to deduct it from the observed osmotic pressure of the protein solution. The term $2y + z - 2x$ we will call the Donnan correction. In this term y and x can be calculated from the measurements of the pH, pH inside being $-\log y$ and pH outside being $-\log x$. z can be calculated from x and y with the aid of the Donnan equation (1)

$$z = \frac{(x + y)(x - y)}{y}$$

since we know that x and y are determined by the Donnan equilibrium. If the value of $2y + z - 2x$ is calculated for different pH of a gelatin chloride solution (of the same concentration of originally isoelectric gelatin which in this case was 1 per cent) ; and if from this value is calculated the osmotic pressure due to this excess of the molar concentration of crystalloidal ions inside the protein solution over that outside, it is found that the curve for the Donnan correction is almost identical with the curve for the observed osmotic pressure. In other words, it turns out that the increase in osmotic pressure of a 1 per cent solution of originally isoelectric gelatin upon the addition of little acid until a maximum is reached, and the diminution of osmotic pressure upon the addition of further acid are not due to any variation in the state of dispersion of the protein, or any other real or imaginary "colloidal" property of the protein, but purely to the fact that protein ions can not diffuse

through the collodion membrane which is easily permeable to crystalloidal ions; as a consequence of which the molar concentration of the crystalloidal ions must always be greater inside the protein solution than outside. What varies with the pH of the gelatin solution is the quantity of the excess of $2y + z$ over $2x$. This follows from the Donnan equation (1) according to which .

$$x = \sqrt{y^2 + yz} \text{ or } 2x = \sqrt{4y^2 + 4yz}$$

while

$$2y + z = \sqrt{4y^2 + 4yz + z^2}$$

Now it is obvious that

$$\sqrt{4y^2 + 4yz + z^2} > \sqrt{4y^2 + 4yz}$$

i.e., the concentration of the crystalloidal ions inside the protein solution $2y + z$ is always greater than the concentration of the crystalloidal ions $2x$ outside, when z is not 0 or ∞.

If we substitute for the term $2y + z - 2x$ of the Donnan correction the identical term

$$\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz}$$

we can visualize why the osmotic pressure is a minimum at the isoelectric point, why it increases with the addition of little acid, reaching a maximum, and why it diminishes again with the addition of more acid.

At the isoelectric point no protein is ionized and z being zero, the whole term

$$\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz}$$

becomes zero. Hence at the isoelectric point the observed osmotic pressure is purely that due to the protein, which is very low on account of the high molecular weight of gelatin.

When little acid, e.g., HCl, is added to the solution of isoelectric gelatin, gelatin chloride is formed and some free acid remains, due to hydrolytic dissociation. Hence both z (the concentration of Cl ions in combination with protein) and y (the Cl ions of the free HCl existing through hydrolysis) increase, but z increases at first more rapidly than y and hence the excess of concentration of ions inside over that of ions outside increases until the greater part of protein is transformed into protein chloride, when the excess of crystalloidal ions inside over those outside reaches a maximum. From then on z increases comparatively little while y increases considerably with further addition of acid, so that z becomes negligible in comparison with y. This explains why the Donnan correction becomes zero again when enough acid is added, and why the observed osmotic pressure becomes as low again as at the isoelectric point.

In the same way it can be shown why the addition of salt has only a depressing effect on the osmotic pressure. Let us assume that there is inside the bag a gelatin chloride solution of pH 3.0 to which NaCl is added. z (the concentration of Cl ions in combination with the gelatin) will not increase with the addition of salt, while y (the concentration of the Cl ions *not* in com-

bination with gelatin) will increase. Hence with the increase in the concentration of the salt the value of

$$\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz}$$

will become smaller, finally approaching zero.

When another salt than a chloride, e.g., $NaNO_3$, is added to a solution of gelatin chloride, we may assume that the gelatin in solution is gelatin nitrate.

Figure 3 gives a comparison of the curves for the observed osmotic pressure and for the Donnan correction. Both curves rise in a parallel way from the isoelectric point reaching a maximum which is 450 mm. H_2O pressure in the case of the observed osmotic pressure and slightly lower in the case of the Donnan correction. The observed osmotic pressure should be higher than the Donnan correction by the osmotic pressure due to the protein solution itself. A difference exists in the values between pH 4.6 and 3.2 but disappears later, and this difference is in all probability the expression of value a, i.e., the osmotic pressure due to the protein itself. The disappearance of this difference at pH below 3.2 is probably due to the fact that an error of one unit in the second decimal of the pH causes a considerable error in the calculations of z, which increases when the pH is too low.

Figure 3 shows that when we correct the observed osmotic pressure for the Donnan effect it follows that the influence of the pH of the acid on the osmotic pressure is entirely or practically entirely due to the excess of the concentration of crystalloidal ions inside the membrane over that outside

FIG. 3.—Observed osmotic pressure curves for gelatin, and Donnan correction.

and that this excess is caused by the Donnan equilibrium. The osmotic pressure of the protein itself is either not altered at all by the addition of acid or if it is altered the effect is too small to be noticeable. There is then nothing left for the "dispersion theory" or for any other of the colloidal speculations to explain. These results were confirmed for crystalline egg albumin and casein by the writer and for edestin by Hitchcock. We now understand why only the valency and not the nature of the ion plays a rôle in the osmotic pressure of protein solutions. The equilibrium equation is one of the second degree when the ion with which the protein is in combination is monovalent while it is of the third degree when the ion is divalent. Only the valency of the ion and not its nature enters into the Donnan equation.

We can therefore summarize these results by stating that the so-called colloidal behavior of protein solutions, as far as osmotic pressure is concerned, is merely the result of an equilibrium condition of classical chemistry which, results in an excess of the concentration of crystalloidal ions inside the protein solution over that of an outside aqueous solution, when the two solutions are
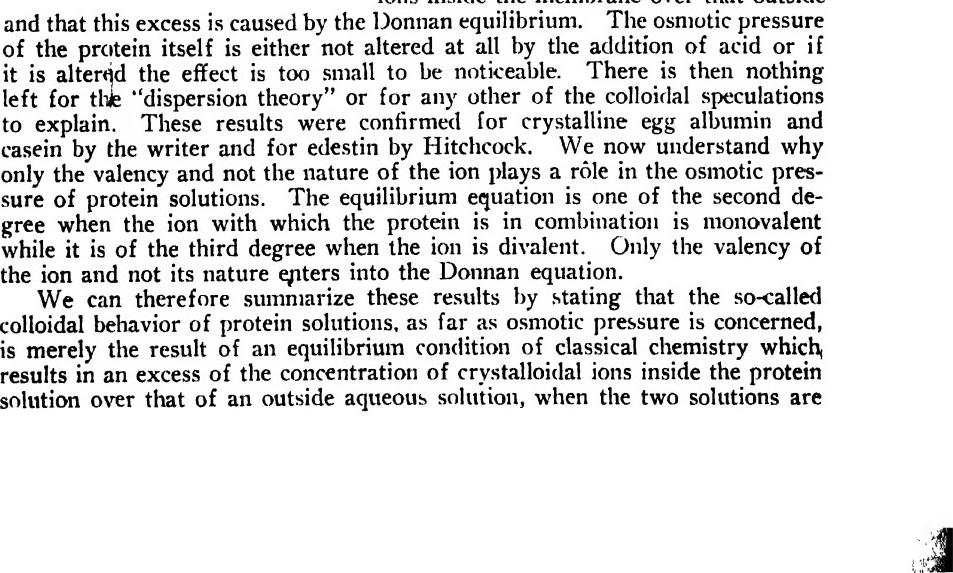
separated by a membrane which is permeable to crystalloidal ions but impermeable to protein ions.   The colloidal behavior of proteins depends therefore entirely on the relative non-diffusibility of protein ions through membranes which are easily permeable to crystalloidal ions.   Since the majority of membranes in plants and animals belong to this class, it can easily be surmised how great a rôle the proteins must play in the regulation of osmotic pressure in the body.

VI

It remains to show briefly why swelling and viscosity of protein solutions are affected in a similar way by electrolytes as is the osmotic pressure.   The answer is that we are dealing in both cases with the same fundamental property, namely, osmotic pressure.

In 1910 Procter * made the ingenious suggestion that the swelling of gelatin might be an osmotic phenomenon and in subsequent papers he and J. A. Wilson put this theory on a quantitative basis by deriving it from the Donnan equilibrium.   They showed that the swelling of a solid gel of gelatin in hydrochloric acid can be explained quantitatively on the basis of the Donnan equilibrium on the assumption that there exists an excess of concentration of crystalloidal ions inside (in this case H and Cl) over the concentration of the same ions outside, and that the excess of osmotic pressure inside the gel over that outside due to this Donnan effect accounts for that share of the swelling which is caused by the influence of the acid.   The agreement of their calculated values with the observed values is excellent.   The writer is inclined to consider Procter's theory of swelling and the proof of this theory by Procter and J. A. Wilson as the most brilliant contribution to the theory of colloidal behavior next in importance only to Donnan's theory of membrane equilibria. There was only one detail left by these authors, namely, to prove the existence of membrane potentials between the gel and the surrounding aqueous solution at equilibrium.   The writer was able to fill this gap and to show that the observed P.D. between gel and surrounding aqueous solution can be calculated with a fair degree of accuracy from the value pH inside minus pH outside with the aid of Nernst's logarithmic formula.

VII

It may seem strange that the influence of electrolytes on the viscosity of certain protein solutions should be explained in the same way, but this seems to be the case.   According to Einstein's formula, the viscosity of an aqueous protein solution is a linear function of the relative volume of the solute occupied in the solution, as expressed in the formula

$$\eta = \eta_0(1 + 2.5\varphi)$$

where η is the viscosity of the solution, η_0 that of pure water, and φ the proportion of the volume of the solute to that of the solution.   If, therefore, the addition of little acid to a 1 per cent solution of isoelectric gelatin increases the viscosity of the solution until a maximum is reached and if the addition of

* A paper by H. R. Procter will appear in Vol. III of this series. *J. A.*

more acid depresses the viscosity again, it follows that the addition of acid changes the relative volume occupied by the gelatin in water. This is only possible by water being absorbed by the protein and the question is how to account for this absorption of water by the protein under the influence of acid. Pauli assumed that the ionized protein surrounds itself with a jacket of water which is lacking in the non-ionized protein. If this were true, all the proteins and amino-acids should show such an influence of acid on the viscosity of their solutions. The writer found that no such influence exists in the case of amino-acids and at least one protein, namely, crystalline egg albumin; if Pauli's assumption were correct, there is no reason why crystalline egg albumin should not show the same influence of acid on viscosity which is found in the case of gelatin. The difference between gelatin and crystalline egg albumin is that the former sets to a solid gel if the temperature is not too high while the latter does not. The formation of a continuous gel in the gelatin solution is preceded by the formation of submicroscopic aggregates which occlude water and which are capable of swelling and these aggregates or precursors of the continuous gel increase in size and number on standing. To test this idea the writer made experiments with suspensions of powdered gelatin in water and found that such suspensions of powdered gelatin had a much higher viscosity than a freshly prepared solution of gelatin. This was to be expected if the influence of acid on the viscosity of proteins is due to the swelling of submicroscopic particles of gel. It harmonizes with this fact that the viscosity of solutions of crystalline egg albumin is of a low order of magnitude, which was to be expected if solutions of crystalline egg albumin contain few or no micellæ. It was found, moreover, that the viscosity of suspensions of powdered gelatin increased under the influence of acid or alkali in the same way as did the swelling of jellies or the osmotic pressure of protein solutions. The viscosities were measured at 20° C. When the suspension of powdered gelatin was melted, it was found upon rapid cooling to 20° C. that the viscosity was considerably lower and that the influence of acid had most disappeared. By these and a number of similar experiments it was possible to prove that the similarity between the influence of electrolytes on the viscosity of gelatin solution and the influence of electrolytes on osmotic pressure due to the fact that the influence on viscosity in such cases is in reality an influence on the swelling of submicroscopic protein particles. This proof was made complete by showing that there exists a Donnan equilibrium between powdered particles of gelatin and a surrounding weak gelatin solution.

VIII

It may not be amiss to illustrate by way of an example why it is that the neglect of measuring the hydrogen ion concentration of protein solutions necessarily lead into errors. In a paper published in 1921 by Kuhn,[3] it was intended to show that different acids of the same valency have different effects on the swelling of gelatin. In order to furnish such a proof it is necessary to start with isoelectric gelatin and to compare the effect of different acids on the swelling of this isoelectric gelatin at the same hydrogen ion concentration of gel, since only in that case have the gels the same concentration

⁸ Kuhn, *Kolloidchem. Beihefte,* **14,** 147 (1921).

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